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Studies on the immunogenicity in swine of influenza A virus hemagglutinin expressed by Venezuelan equine encephalitis virus-like replicon particles

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CHAPER 1: GENERAL INTRODUCTION

Since its isolation from pigs in 1930, influenza A virus has had devastating effects on the swine industry[102]. In the United States, at least 25% of pigs have been found to be positive for antibodies against swine influenza virus at slaughter[23]. Economic loss can be attributed to an increase in the time needed for pigs to reach market weight[80]. Although several vaccines are currently on the market to combat influenza infection in swine, none are able to protect against all subtypes of the virus. New virus reassortants continue to be isolated from the swine population, demonstrating the need for an improved method of vaccination against influenza virus in pigs[62, 65, 124].

The focus of this thesis is to describe work done towards the development of a new vaccination method for influenza in swine. Chapter 2 provides a general overview of influenza A virus including current vaccination methods in pigs. Chapters 3 and 4 are written in journal article format and describe the author's research experiments. Chapter 5 provides general conclusions for the author's thesis.

CHAPER 2: LITERATURE REVIEW

Section 1: Introduction - Influenza A Virus

Influenza A virus is known to infect a wide range of species including humans, swine, horses, tigers, dogs, whales, mink, and other animal species[16, 73, 118]. Historical accounts of influenza epidemics date back to the 14th and 15th centuries, but swine influenza A virus was first isolated in 1930 from pigs, and human influenza A virus was first isolated in 1933 from ferrets[88, 102, 107]. Since that time, much work has been done to characterize the virus and determine its mechanism of pathogenesis; however, more work is needed especially in the area of vaccine development.

Disease caused by influenza A virus in mammals is characterized by the rapid onset of clinical symptoms including fever, coughing, dyspnea, lethargy, and pneumonia. Recovery following infection is rapid in the absence of secondary pathogens. Occasionally, viral pneumonia is complicated by secondary infection and can lead to death[23]. Several major influenza pandemics have occurred in the human population throughout history, including 1918, 1957, 1968 and 1977[116]. Currently, various vaccines are available to combat influenza A virus infections in humans and animals including swine and horses. However, due to the virus's ability to undergo rapid change in its antigenic properties through antigenic drift and shift, a universal vaccine that is effective against many or all subtypes of the virus has yet to be developed[23, 82, 100]. The following sections will briefly outline current classification of the virus, its mechanism of pathogenesis, host immune response to the virus, and current methods of vaccination.

Section 2: Characterization and Classification

Influenza A virus is a member of the family *Orthomyxoviridae*, genus influenza A virus[133]. It is a pleomorphic, enveloped virus with helical symmetry[73]. The genome of influenza A is

approximately 13.6 kbp in size and consists of eight single-stranded, negative sense RNA segments[12, 104]. Virus particles range in size from approximately 80 to 120 nm in diameter[73]. The eight gene segments in each virion encode a total of eleven known proteins: three viral polymerase subunits (PB2 from segment 1, PB1 from segment 2, and PA from segment 3); PB1-F2 from segment 2, a recently discovered protein that is thought to be involved in the induction of host cell death; hemagglutinin (HA) from segment 4, and neuraminidase (NA) from segment 6, both surface glycoproteins; nucleoprotein (NP) from segment 5 which binds and surrounds the viral RNA segments; matrix 1 (M1) from segment 7 which makes up a majority of the viral protein shell; matrix 2 (M2) from segment 7, a transmembrane protein which acts as a proton channel; nuclear export protein (NEP, formerly known as NS2) from segment 8; and non-structural protein 1 (NS1) from segment 8 which plays a role in evasion of the host immune response[10, 13, 131, 133].

Influenza A virus is one of four genera in the family *Orthomyxoviridae*, and is differentiated from influenza B virus, influenza C virus, and thogotovirus based upon several characteristics including differences in NP and M1 protein identity, number of gene segments in each virion, and differences in host range[73, 133]. Currently, sixteen different HA and nine different NA subtypes have been isolated from influenza A viruses in wild birds[26]. Many of these HA and NA subtypes have only been found in avian species. In humans, subtypes H1N1, H2N2, and H3N2 have been the predominate causes of disease, with subtypes H5N1, H7N7 and H9N2 causing fewer, more isolated cases of human disease[59].

HPAI vs. LPAI Viruses:

As all known subtypes of influenza A viruses are found in wild birds, influenza A viruses have been categorized as either high pathogenic avian influenza (HPAI), low pathogenic avian influenza (LPAI), or nonpathogenic avian influenza (NPAI)[2, 39, 108]. Many factors may play a role in determining viral pathogenicity, as several influenza viral RNA (vRNA)

segments have been shown to affect host range and virulence[108]. However, it has been demonstrated that the amino acid sequence at the cleavage site of HA (discussed further in Section 3, below) is one major determinant of virulence. HPAI viruses contain multiple basic amino acid residues at their HA cleavage site, while LPAI and NPAI typically have one arginine or lysine found at the carboxyl terminus of HA1 and one glycine at the amino terminus of HA2[39, 108]. To date, the only naturally occurring HPAI isolates have contained HA subtypes H5 or H7, and these HPAI viruses have occasionally crossed the species barrier into humans and other animals[108, 118].

Drift and Shift:

Influenza viruses are known for their ability to rapidly change their antigenic properties, making it difficult to develop an efficient vaccination strategy against them. These rapid antigenic changes are carried out by two main mechanisms, antigenic drift and shift. Antigenic drift refers to slight changes in the amino acid sequence of an antigenic domain within a viral gene. Drift is commonly seen in the HA and NA genes of influenza viruses, and this is important as these two proteins have been shown to have an effect on host range and virulence of a virus[43, 94]. Antigenic shift refers to the swapping of entire gene segments (genetic reassortment) between different virus strains during a coinfection of two or more viruses. Any of the eight viral RNA segments can undergo shift, and segments can be shared between species[109].

Swine Influenza A Viruses:

Three different subtypes of influenza A virus predominate in swine populations today, H1N1, H1N2, and H3N2, with genes originating from swine, human and avian influenza strains[62]. However, due to genetic reassortment, new strains are being isolated from swine herds throughout the world including the United States, Canada, and Europe[47, 62, 65, 124].

Section 3: Disease and Pathogenesis

Aquatic avian species are the natural reservoir of influenza A viruses[73]. These viruses do not generally cause clinical disease in wild fowl, but the virus is transmitted to other animal species from aquatic birds where it can cause disease[78]. The virus typically replicates in intestinal epithelial cells of wild birds and is excreted into the environment in the feces where it can be transmitted to other animals, importantly domestic birds and livestock[73, 78].

Swine influenza A virus typically causes an acute, respiratory illness that can affect pigs of any age. Symptoms may include fever, coughing, rhinitis, pharyngitis, and pneumonia, with rapid onset and recovery. In a herd, it is common to see up to 100% morbidity, but low mortality rates[22, 23].

Attachment:

Influenza virus infection begins when the virus binds to host cell surface receptors (sialyloligosaccharides) via viral hemagglutinin (HA)[112, 137]. The HA glycoprotein exists on the surface of each virion as a homotrimer. Specific host cell proteases cleave the HA glycoprotein (designated HA0) at a conserved arginine residue into two subunits, HA1 and HA2, which remain linked together by a disulfide bond[96, 108, 110]. For low or nonpathogenic viruses, HA is cleaved extracellularly by trypsin-like proteases that are expressed only by a few cell or tissue types, preventing the systemic spread of the virus[108]. The HA of pathogenic viruses is cleaved intracellularly by subtilisin-like proteases, enabling them to immediately infect other cells upon budding from their host cell[108]. Cleavage of HA0 facilitates viral entry into host cells by exposing the sialic acid binding pocket on HA1 and membrane fusion peptide on HA2, and allowing HA to undergo the conformational change necessary to be activated by the low pH of cellular endosomes[69, 108].

Receptor Strength and Specificity:

Receptor binding strength and specificity of HA play a large role in determining the host range of a specific virus. The position and number of glycans on an HA molecule can vary from virus strain to strain[42, 68]. This variation in glycosylation pattern can influence the virus's host range, and can also play a role in the virus's requirement for neuraminidase (NA)[94]. Neuraminidase, influenza's other major surface glycoprotein, also recognizes host cell sialyloligosaccharides, but acts in opposition to HA, cleaving linkages between sialic acid and its adjacent sugar residue in order to aid in the release of progeny virions during budding[33, 105]. Work done by Mishin et al[68] has suggested that specific HA glycosylation patterns can reduce a virus's need for NA activity.

It has also been determined that a virus's receptor binding specificity is strongly correlated with the amino acid at position 226 of the HA protein. If glutamine is located at position 226, then HA will preferentially bind to 5-N-acetylneuraminic acid containing α–(2,3) linkages. However, if leucine is located at position 226, then HA will preferentially bind to receptors containing α–(2,6) linkages[136, 137]. Human influenza A viruses typically prefer receptors with α–(2,6) linkages, as human epithelial cells lining the trachea possess primarily NeuAcα2,6Gal sialyloligosaccharides, while avian viruses prefer receptors with α–(2,3) linkages as NeuAcα2,3Gal sialyloligosaccharides are predominant in the intestinal tract of wild birds[112]. Pigs, however, have been shown to possess both NeuAcα2,3Gal and NeuAcα2,6Gal sialyloligosaccharides on the epithelial cells lining the trachea, suggesting that they can efficiently support infection of both avian and human strains of influenza A virus[43].

Entry:

After the virus has bound to its host receptor, it is taken into host cell endosomes[139]. The low pH of the endosome causes HA to undergo a conformational change that leads to

fusion of the endosomal membrane with the viral envelope[39]. Matrix 2 is a proton channel spanning the viral membrane that allows the inner portion of the virion to be exposed to the low pH of the endosome, causing the viral ribonucleoproteins (vRNPs) to detach from the M1 viral protein shell and move into the cell cytoplasm[39]. The vRNPs are then transported into the host cell nucleus, where viral RNA undergoes both transcription to form viral mRNA for protein synthesis, and replication to make copies of the viral RNA (vRNA) segments to be packaged in new virions[34, 74].

RNA Replication and Protein Synthesis:

The vRNA polymerase responsible for both transcription of viral mRNA and viral genome replication is a complex of three proteins: polymerase B1 (PB1), polymerase B2 (PB2), and polymerase A (PA)[40]. Polymerase B1 is believed to be responsible for RNA-dependent-RNA-polymerase activity, while PB2 is thought to be involved in mRNA transcription initiation through capping of viral mRNA[5, 9, 74]. The role of the PA protein is less clearly understood. Cloned complementary DNA (cDNA) studies have demonstrated that PA expression is correlated with a degradation of other proteins, both cellular and viral, but whether this role is significant in an actual viral infection has yet to be determined[98]. Recent work done by Huarte et al[41] showed that viruses containing mutations in the PA gene, particularly at threonine 157, had altered PA protease activity and overall viral complementary RNA (cRNA) synthesis.

The viral polymerase complex must transcribe viral mRNA to make proteins, and must also make full length cRNA in order to replicate viral vRNAs for packaging into new virions. It has been determined that transcription into mRNA is initiated with capped RNA priming using host cell mRNA, whereas transcription of cRNA to make full length vRNA is primer-independent[35, 86, 87, 103]. All eight influenza virus vRNAs consist of a coding region flanked on each end by untranslated regions (UTRs) containing sequences that are highly

conserved between vRNAs and between different virus strains[58]. Studies have shown that sequences within the 5' UTR of the viral mRNA segments regulate the preferential translation of viral mRNAs over host cell mRNAs[30, 85]. It has also been shown that the host cell RNA-binding protein GRSF-1 interacts with portions of the 5' UTR on viral mRNAs to influence viral protein synthesis, although the exact mechanism of this interaction is unknown[85].

Once vRNPs are in the nucleus, viral transcription to make 5'-capped, 3'-polyadenylated mRNAs begins[4]. Upon viral infection of cells, host mRNA translation is blocked at both the initiation and elongation steps, as cellular mRNA remains bound to ribosomes[48]. One host cell protein that has been shown to play a significant role in viral mRNA translation is $p58^{IPK}$. Upon viral infection, several interferon-induced host cell proteins begin to work to combat viral infection, including PKR. $p58^{IPK}$ inhibits PKR (explained further in Section 4, below), aiding in viral mRNA transcription[32]. When a sufficient amount of viral mRNA has been produced, viral transcription is downregulated, and viral genome replication commences. Influenza virus nucleoprotein (NP) plays an important role in this switching from mRNA production to cRNA and vRNA production by a mechanism thought to involve the direct interaction of NP with the PB2 subunit of the polymerase complex[8].

While cRNA and vRNA are being replicated in the nucleus, viral mRNA is being translated into proteins in the cytoplasm in preparation for assembly of new virions. Matrix l protein, the major inner structural protein of influenza virus, also plays an important role in the progression from genome replication to virus assembly. Once M1 mRNA is translated into protein in the cytoplasm, the protein enters the nucleus and binds to newly formed ribonucleoproteins (RNPs) to facilitate their exit into the cytoplasm, and to prevent them from re-entering the nucleus[60]. In addition to the M1 protein, it has been suggested that the nuclear export protein (NEP) plays a critical role in the export of newly formed RNPs from

the nucleus to the cytoplasm[77]. With all viral proteins now in the cytoplasm, the process of virus assembly and budding is able to proceed.

Control of Host Gene Expression:

During influenza virus infection, host cell gene expression is drastically changed. Studies using DNA microarray analysis of over 4,500 cellular genes demonstrated that many host genes are regulated during influenza virus infection both dependently and independently of viral replication. Importantly, some genes that are downregulated include genes involved in progression of the cell cycle, several transcriptional regulators, and genes involved in the ubiquitin pathway, while the metallothionein genes are among those that are upregulated[31]. The exact mechanisms used by influenza virus in controlling gene regulation have not been determined, but suggestions as to the probable cause of this altered gene expression included transcriptional regulation by viral proteins as well as the shift in stability of host cell mRNA following viral infection[31]. One viral protein that has been shown to play a role in the control of host protein expression is NS1. Work done by Fernandez-Sesma et al[25] demonstrated that the presence of NS1 protein in influenza virus prevents the production of interferon-alpha (IFN-α) and interferon-beta (IFN-β), and inhibits the maturation of dendritic cells, thereby downregulating the T-cell response.

Virion Assembly and Budding:

During virion assembly in host cells, all of the necessary components must be present in each virion in order for the virus to successfully infect new cells. A sufficient amount of HA is necessary on the surface of each virus particle to facilitate attachment and entry into a host cell. Studies have demonstrated that during virus assembly, HA molecules cluster together in host cell lipid rafts. It has been suggested that this clustering allows for the necessary amount of HA to be expressed on each virion for infection of new host cells[114].

For the eight vRNAs, it has been reported that cis-acting packaging signals are found within the UTRs of the segments, and that these signals extend into the coding region of the segments[58, 71]. It has been suggested that the PB2 vRNA segment may be involved in the assimilation of the other seven vRNA segments into budding virions[71]. It has also been shown that vRNAs are specifically arranged in new virions. Seven segments surround the eighth segment in a circular or cubodial pattern, and all eight segments are associated with the budding end of a new virion[75].

As new virions begin to bud out of their host cell, NA recognizes host sialic acid receptors and functions to cleave α-2,3 or α-2,6 ketosidic linkages between HA and its cell surface sialic acid receptor allowing the new virions to completely bud out of the plasma membrane and infect new host cells[69].

Section 4: Immune Response

Immunopathology:

After influenza virus enters a new host, virally infected cells as well as antigen presenting cells that have encountered viral proteins are stimulated to produce a variety of cytokines, leading to the induction of cytokine-regulated proteins. Interferons α and β (IFN- α, IFN-β) stimulate the production of protein kinase, PKR (also know as DAI, dsI or P1/eIF-2 kinase, P68), which is then activated by dsRNA to phosphorylate eukaryotic initiation factor 2 alpha (eIF-2α), leading to the downregulation of protein synthesis in the host cell[7, 27, 49, 119]. Influenza virus has developed a mechanism to combat this host cell strategy of attempting to shut down viral RNA synthesis by interacting with the host protein $P58^{IPK}$ using a not yet characterized mechanism that inhibits PKR from phosphorylating eIF-2α, allowing viral protein synthesis to occur[32].

Studies in pigs have shown that IFN-α, tumor necrosis factor- α (TNF-α), and interleukin-1 (IL-1) are three cytokines that play an important role in the host's immune

response to influenza virus infection. All three cytokines are able to induce non-specific antiviral effects as well as fever, and TNF-α and IL-1 have been shown to play an important role in the migration of large numbers of neutrophils and monocytes to the lungs as part of the inflammatory response[126]. Based on studies of cytokine expression in pigs following influenza virus challenge, it has been suggested that the virus induces a strong cytokine response in the lungs. This cytokine response is an effect of viral infection, resulting in a more severe respiratory infection than what is seen with other swine respiratory viruses including porcine respiratory coronavirus and some isolates of porcine reproductive and respiratory syndrome virus[125]. A study demonstrated that pigs vaccinated with either a heterologous or homologous vaccine that were not completely protected from virus challenge still had markedly reduced levels of IFN-α, TNF-α, IL-1, IL-6 and IL-8 in their lungs compared to unvaccinated, challenged pigs[127]. In the same study, pigs vaccinated and completely protected after challenge showed a reduction in neutrophil infiltration to the lungs and even lower levels of IFN-α, IL-6 and IL-8 than vaccinated pigs that were not protected, while levels of TNF- α and IL-1 were approximately the same in both groups. This demonstrates the differences in cytokine profiles between vaccinated and unvaccinated pigs.

Although virus virulence has been shown to be affected by the strength and specificity of influenza HA binding to its host cell receptor, other mechanisms of virulence relating to the virus's ability to overcome host immune response have been demonstrated. Work done by Seo et al[101], suggested that the high level of virulence expressed by H5N1 influenza viruses may be due to the ability of their NS1 gene to overcome the antiviral effects of various host cell cytokines including IFN-α, IFN-γ, and TNF-α. Pathogenic H5N1 viruses were shown to contain a glutamic acid residue at position 92 in the NS1 gene, whereas nonpathogenic viruses contain an aspartic acid residue at this position. Pigs inoculated with a recombinant virus containing the NS1 gene from the H5N1 virus showed an increased viral load in the lungs, increased body temperature, and increased length of viral shedding when

compared to pigs inoculated with an H1N1 virus containing the H1 NS1 gene[101]. Work done by Li et al[57] suggested that the amino acid residue at position 149 of the NS1 gene is responsible for virulence phenotype in chickens.

Humoral and Cell Mediated Immune Response:

In newborn piglets, immunoglobulin acquired from their dam through colostrum in the first 24 to 36 hours after birth acts to protect them until their adaptive immune responses have developed[72]. This acquired or maternally derived antibody (MDA) can have a number of effects on the immune response of piglets exposed to a variety of pathogens including influenza virus. Work done by Loeffen et al[61] examined the effects of MDA on influenza virus excretion, antibody production and T-cell responses in piglets experimentally challenged with a homologous H1N1 virus. It was shown that piglets with MDA were incompletely protected from homologous challenge, and they shed virus longer and demonstrated an inhibited immune response against a second infection when compared to piglets without MDA, although these results may be relevant only for piglets with similar levels of MDA as seen in this study. A study performed by Kitikoon el al[52] demonstrated that MDA does not protect piglets from heterologous virus challenge, and may actually enhance disease.

Several studies have been conducted to evaluate the specific antibody responses of pigs after infection with various strains of influenza virus. Work done by Kim et al[50] evaluated the antibody response of 5-week-old pigs experimentally infected with either an H1N1 or H3N2 SIV isolate. Infected pigs developed antibodies to the virus they were infected with by 7 days post infection (PI), whereas pigs vaccinated with a commercial bivalent vaccine did not show an antibody response by hemagglutination-inhibition (HI) assay until two weeks after a second vaccination (4 weeks after first vaccination). Specific antibody profiles of experimentally infected pigs were also examined. It was shown that IgM

antibodies specific for HA were present in all infected pigs at 7 days PI, whereas IgG antibodies specific for HA, although present in some pigs by 7 days PI, did not show up in all pigs until 14 days PI and could be detected until termination of the study (28 days PI). The presence of antibodies against several other influenza proteins was also evaluated. No IgM antibodies specific for NA or M1 proteins could be detected by western blot in any of the pigs throughout the study, but IgG antibodies specific for NA and M1 were detected in most pigs by 14 days PI and all pigs at 28 days PI. IgM antibodies specific for NP, NS1 and NEP (NS2) proteins were detected in all pigs by 7 days PI, and could be detected in most pigs until 28 days PI. IgG antibodies specific for NP, NS1 and NEP (NS2) proteins were detected in all pigs from 7 days PI until 28 days PI.

Work done by Larsen et al[53] evaluated the serum and mucosal antibody responses of pigs to experimental infection with an H1N1 SIV isolate. It was shown that after infection, virus-specific serum IgG levels were two to four times higher than serum IgA levels at all time points during the study (7 to 56 days post initial infection). The opposite was observed in both the upper and lower airways, with virus-specific IgA levels being higher than IgG levels in both areas throughout the study. Pigs were infected for a second time 42 days after initial infection with the homologous virus, and neither virus-specific IgG nor IgA levels increased dramatically in either the serum or lower airway, but did increase substantially in the upper airway. The location of IgG and IgA producing cells as well as IFN-γ producing cells was also evaluated. It was shown that the primary location of IgG and IgA producing cells was the nasal mucosa, with more IgA-producing cells than IgG-producing cells being detected. The primary location of IFN-γ producing cells was the tracheobronchial lymph nodes and spleen.

Other work has been done to evaluate the differences in both humoral and cellular immune responses between pigs infected with heterologous versus homologous strains of influenza virus. Heinen et al[36] evaluated the humoral and cellular immune responses of pigs

experimentally infected at two time points with either homologous (H3N2) or heterologous (H1N1 and H3N2) virus strains. After first infection with either H1N1 or H3N2, both groups of pigs developed HI titers against their challenge virus, but not against the heterologous virus. After a second infection, the group of pigs initially infected with H1N1 and then infected with the heterologous H3N2 SIV strain (group Het-I) developed strong HI titers against the H3N2 strain, and they also showed a slight increase in HI titers against the H1N1 strain. An M2e-specific ELISA was also used to show that after second infection, pigs in the Het-I group developed high levels of serum IgG against the M2e protein. This high level of response was not seen in the group of pigs infected both times with H3N2 SIV (group Hom-I). The cellular immune response was characterized by an influx of neutrophils into the lungs by 2 days after first infection in both groups. After second infection, the level of infiltration decreased in group Het-I and was not seen in group Hom-I. Also after approximately 2 days following the first infection, both groups had an increase in natural killer (NK) cells, at 4 to 11 days an increase in T-helper (Th) cells, and at 8 days a large increase in cytotoxic lymphocytes (CTLs) corresponding to a drop in NK cells. After the second infection, a further increase in CTLs was observed in group Het-I, but not in group Hom-I, therefore it was suggested that in order for a vaccine to induce heterologous protection against various influenza virus strains, it should elicit a CTL ($CD8^+$ T-cell) response.

Section 5: Vaccination

The development of a safe, efficacious vaccine strategy to combat influenza virus infections in both humans and animals has posed a problem for decades. As early as the 1950s researchers saw the difficulty in developing a universal influenza vaccine due to the virus's ability to undergo antigenic drift and shift[138]. Since that time, much work has been done in an attempt to improve the current influenza vaccination methods.

Inactivated Whole and Subunit Vaccines:

One of the early methods of vaccinating against influenza virus in humans used whole, formalin inactivated virus particles grown in embryonated chicken eggs[97]. Inactivated vaccines are also used to vaccinate swine against influenza A virus. A study conducted by Heinen et al[38] evaluated the immune response of a group of pigs vaccinated with a commercial spilt vaccine (primary component of the spilt vaccine was HA protein) from a human H3N2 virus then challenged with a swine H3N2 virus currently circulating in the swine population versus a group of pigs inoculated and then challenged with the same swine H3N2 virus. The spilt vaccine induced a strong IgG antibody response, but induced a weak local IgA antibody and cellular response, and provided sub-optimal protection against the challenge virus, as viral shedding was reduced but not eliminated. In the group of pigs previously infected then challenged with the swine H3N2 virus, a higher IgA antibody and cellular response was observed, and pigs were completely protected against homologous challenge. This work suggests that in order for a vaccine to confer complete protection against influenza A virus, both a cellular and humoral immune response must be elicited.

Live-Attenuated Vaccines:

Live-attenuated vaccines may be able to more closely mimic natural infection, and therefore induce a better cell-mediated immune response than inactivated vaccines. Work done by Richt et al[92] demonstrated that pigs vaccinated with a modified-live influenza virus vaccine made by a deletion in the NS1 gene of an H3N2 virus were completely protected against homologous challenge. The pigs were only partially protected against heterologous challenge with an H1N1 influenza virus.

There have also been several recent studies examining the use of live-attenuated vaccines derived from a combination of several different virus strains for the purpose of inducing a more efficacious heterologous protection against various strains of influenza

virus, in particular the recently emerging HPAI H5N1 strains. Desheva et al[19] demonstrated that a reassortant live-attenuated virus constructed from an H5N2 and a cold-adapted H2N2 virus could protect mice from a lethal challenge against multiple avian H5N1 virus strains, and mice demonstrated a high level of virus-specific mucosal IgA antibodies. The reassortant virus was also formalin inactivated and given to mice intramuscularly, and a similar level of protection was observed, however, a stronger serum-neutralizing IgG antibody response was observed in the formalin inactivated group, with less of an IgA antibody response.

Recombinant Protein/ Viral Vector Vaccines:

Recombinant protein technology is a relatively new area in vaccine development. Several different types of recombinant protein vaccines have been studied to combat influenza A virus infection, such as DNA vaccines in the form of plasmids delivered directly[81], plasmids in a carrier organism[129], proteins expressed in recombinant viruses[128], and proteins expressed in replicon particles[120].

DNA vaccines in the form of plasmids delivered directly into the skin or muscle tissue have been widely studied. Several studies by different research groups have examined the use of DNA vaccines against influenza viruses in pigs, with both positive and negative results being observed. Work done by Macklin et al[64], demonstrated that pigs vaccinated with plasmid DNA expressing influenza HA coated onto gold particles and delivered via gene gun to the epidermis or tongue epithelium was able to elicit a protective immune response following homologous challenge. The DNA vaccine did not completely protect against challenge, but it reduced the length and severity of viral shedding.

Work is also being conducted by other researchers to study the use of DNA expressing influenza proteins as a primer vaccine followed by a boost using a conventional or

recombinant vaccine[79]. Heinen et al[37] demonstrated that pigs vaccinated with a DNA vaccine expressing either influenza M2 protein alone or M2 and nucleoprotein (NP) enhanced disease in pigs after challenge, suggesting that antibodies against influenza M2 may not aid in protection against influenza disease. However, studies in mice by Tompkins et al[122] suggest that the M2 protein may be good target for providing heterologous protection against multiple influenza strains, since the M2 protein is more highly conserved than either the HA or NA proteins.

Viruses from several different families have been evaluated for their use as expression vectors in vaccine studies including pseudorabies virus, a member of *Herpesviridae*[121]; vesicular stomatitis virus, a member of *Rhabdoviridae*[29]; several different serotypes of adenovirus, all members of *Adenoviridae*[115]; Newcastle disease virus, a member of *Paramyxoviridae*[130]; and several different alphaviruses, all members of *Togaviridae*[91]. The following paragraphs will discuss some of the work that has been done regarding the use of viral vectors as vaccines against influenza A virus.

Adenoviruses are linear, double stranded DNA viruses that have been widely studied in vaccine development as foreign protein expression vehicles. Originally studied as vectors for gene therapy, their ability to elicit a strong host immune response as well as their wide tissue tropism made them desirable as vaccine vectors[21, 115]. Several different adenovirus vectors have been studied for use against a variety of pathogens including human immunodeficiency virus (HIV) in nonhuman primates and humans[1]; Ebola virus and *Bacillus anthracis* in humans[1]; *Yersinia pestis* in mice[14]; and influenza A virus in mice, chickens and swine[28, 134].

Work done by Wesley et al[128] demonstrated that pigs vaccinated one time intramuscularly with an Ad5 recombinant adenovirus vector expressing influenza A virus

(H3N2) hemagglutinin (HA) alone, or expressing HA and nucleoprotein (NP) together, were protected against challenge with a closely related H3N2 influenza virus. Ad5 vectors expressing NP alone, however, did not show the same level of protection. It has also been shown that piglets with maternal antibodies against influenza H3N2 could be protected against homologous challenge after vaccination with recombinant Ad5 vector expressing influenza HA and NP and boosted 3 weeks later with a commercial vaccine (End-FLUence 2)[135].

One of the major problems associated with human adenovirus vector vaccines is the development of a strong neutralizing antibody titer against adenovirus proteins, therefore preventing the vector from effectively expressing protein after a single administration[111]. This problem may be due, in part, to the fact that many adenovirus vectors, including those tested for animal vaccines, are derived from human adenovirus serotype 5 (Ad5). In order to overcome this problem, researchers are exploring the use of nonhuman adenoviruses as vectors, as well as producing chimeric adenovirus vectors from two or more different serotypes[1, 3, 24, 95].

Newcastle disease virus (NDV) is a single-stranded, negative sense RNA virus that encodes six genes[130]. Researchers have recently demonstrated that chickens vaccinated with a recombinant NDV expressing the HA gene from an avian influenza A virus subtype H5N2 were protected from a lethal challenge of both NDV and the homologous H5N2 influenza virus[130]. The vaccine was also able to reduce the level of viral shedding in chickens.

Alphaviruses are single-stranded, positive sense RNA viruses with small genomes approximately 12Kb in length[91]. Their genome is composed of two open reading frames (ORFs) with four nonstructural proteins on the 5' end of the genome followed by a 26S promoter and five structural proteins on the 3' end[91]. In most alphaviruses, the genomic

RNA packaging signal is located in the coding region of the first or second non-structural protein[91]. Several different alphaviruses have been engineered for use as virus-like replicon particle (VRP) vaccines including: Sindbis virus vector against measles in non-human primates[84]; Semliki Forest virus vector against influenza virus in mice[6]; and Venezuelan equine encephalitis (VEE) virus vector against Lassa and Ebola viruses in guinea pigs[89], against Simian immunodeficiency virus in macaques[18], against human immunodeficiency virus in humans[67], and against influenza A virus in mice and humans[17].

Virus-like replicon particle vaccines derived from alphaviruses have never been tested for use in pigs. The work described in Chapter 3 of this thesis describe studies that evaluate the ability of a VRP vaccine derived from Venezuelan equine encephalitis (VEE) virus to elicit an immune response in pigs against a gene of interest, influenza A virus hemagglutinin (HA). Because VRPs had never been tested in pigs, concentrations of VRPs used as well as dosing schedule were based upon previous work using VRPs in other animal species including mice[11, 17, 54-56], guinea pigs[89], and nonhuman primates[18]. Chapter 4 describes work done to evaluate the ability of an HA protein lysate vaccine (produced *in vitro* by infecting Vero cells with VRPs expressing the influenza HA gene) to elicit an immune response in pigs against the HA protein.

CHAPTER 3. GENERATION OF AN IMMUNE RESPONSE AGAINST INFLUENZA HEMAGGLUTININ IN PIGS VACCINATED WITH AN ALPHAVIRUS REPLICON

Introduction

The pork industry has struggled with disease caused by influenza A virus for decades. Since its isolation from pigs in 1930, much work has been done to develop an effective vaccination strategy against the virus, but due to its highly mutagenic properties the virus is continuously evolving, allowing it to persist as a common respiratory pathogen in swine[102]. Current vaccination methods are unable to provide protection against all newly emerging virus strains, and have been shown to be inadequate at protecting pigs in the presence of maternally derived antibody (MDA), therefore new methods of vaccination must be explored[52, 62, 65, 124].

Recombinant proteins expressed via viral vectors are currently being evaluated for their use in the area of vaccine development. Due to the simplistic nature of the alphavirus genome, several members of this genus have been evaluated for their use as expression vectors for vaccination against a variety of pathogens including influenza A virus[6, 67, 84]. Venezuelan equine encephalitis (VEE) virus is a member of the genus alphavirus, family *Togaviridae*. The genomic structure of VEE consists of single-stranded, positive-sense RNA approximately 11.4 kbp in length[132]. There are four nonstructural (NS) proteins located at the 5' end which, upon infection in a host cell, are translated into two polyproteins, P123 and P1234[70]. Nonstructural protein 2 (nsP2) acts to cleave these polyproteins into four individual NS proteins (nsP1 – nsP4) which make up the viral replication complex that synthesizes both full-length copies of the VEE genome as well as subgenomic viral RNAs[70]. A 26S subgenomic promoter is located after the NS proteins and functions to control expression of viral structural proteins (capsid-E3-E2-6K-E1) on the 3' end of the genome[132].

Venezuelan equine encephalitis virus is an arthropod-born pathogen that has been known to cause severe disease outbreaks in humans and equine species in Mexico, Central America and the northern part of South America since the early 1900s, and it is also known to infect other animal species including deer and swine[20, 106, 132]. A live attenuated VEE vaccine strain, TC-83, was developed in 1961 by passing the virulent Trinidad donkey (TRD) strain 83 times in fetal guinea pig heart cells. The location of the genetic changes that provide the attenuation of TC-83 have been found to be located in the 5'-noncoding region and E2 gene of the virus[51, 132].

Venezuelan equine encephalitis virus has been shown to target lymphoid tissue, specifically dendritic cells during infection, making the virus an ideal candidate for a vaccine vector[63]. Vectors derived from VEE are of two basic types, double promoter vectors and replicon vectors[90]. Double promoter vectors are constructed by inserting a second 26S promoter either immediately proceeding the original promoter or immediately following the E1 gene. A gene of interest can then be inserted directly downstream of the new promoter, allowing the expression of the gene of interest as well as the production of viable VEE virions in a host[17, 90]. Basic construction of replicons is accomplished by deleting the VEE structural genes downstream of the 26S promoter and inserting in their place a gene of interest. During construction of the replicon particles, the structural genes are provided *in trans*, via helper RNAs[90]. Replicon particles derived from VEE have been evaluated as vaccine vectors in a variety of animal species as well as humans, and clinical trials are currently being conducted using VEE replicons against human immunodeficiency virus[67].

Virus-like replicon particles (VRPs) derived from VEE have not yet been tested in pigs for their use as vaccine vectors, therefore we performed a series of experiments to determine whether or not VRPs would be able in induce an immune response against influenza A/Wyoming/03/2003 hemagglutinin (HA) in pigs. VRPs were provided by AlphaVax, Inc. (Research Triangle Park, North Carolina). VRPs were derived from two

different VEE strains, TC-83 described above and V3014, an attenuated VEE strain derived from the Trinidad donkey strain, with single amino acid changes in each of the E1 and E2 genes[123]. Strain TC-83 is considered a Biosafety Level 2 agent (BL-2) and VRPs derived from this strain may be constructed under BL-2 conditions. Although V3014 contains attenuating mutations, it is still considered a select agent; therefore VRPs derived from this strain must be constructed under Biosafety Level (BL-3) conditions.

Two separate trials were conducted to evaluate the ability of VRPs to elicit an immune response against influenza HA in pigs. In Trial I, we used VRPs derived from V3014 to vaccinate pigs intramuscularly with different concentrations of the VRP. Our goal was to determine if VRPs expressing the hemagglutinin (HA) protein of influenza A/Wyoming/03/2003 would be able to elicit an immune response against the HA protein in pigs. Due to unexpected results from Trial I, we were unable to determine if the VRPs could elicit an immune response against HA in pigs. Therefore, in Trial II our first objective was to determine if the VRPs could elicit an immune response against HA protein in pigs. We used VRPs derived from either V3014 or TC-83 to vaccinate pigs intramuscularly or intranasally. We also wanted to determine if differences could be detected in the immune responses to the HA protein in pigs vaccinated with VRPs derived from VEE strain V3014 compared to VEE strain TC-83. Lastly, we evaluated the difference between intramuscular (IM) versus intranasal (IN) routes of vaccination.

Materials and Methods

Construction of Virus-like Replicon Particles:

Replicons were constructed by removing the structural genes of VEE and inserting in their place a spacer region (nonspecific RNA sequence), followed by an internal ribosome entry site (IRES), then the influenza A hemagglutinin (HA) gene of A/Wyoming/03/2003 immediately downstream of the 26S promoter[46]. Replicons used in the negative control pigs

were constructed in the same manner, but the Gag gene of human immunodeficiency virus (HIV) was inserted after the IRES in place of the influenza HA gene. Many different viruses encode IRES elements in their genomes in order to selectively recruit host cell 40S ribosomes to translate viral mRNA into proteins[99]. By inserting the IRES element upstream of the influenza HA gene, production of HA protein in host cells could be selectively increased, with the goal of eliciting a stronger immune response to the HA protein in pigs[46]. During assembly of VRPs, VEE structural genes were provided *in trans* via two separate helper plasmids, one containing the capsid gene of VEE and the other containing the E3-E2-6K-E1 genes of VEE in order to decrease the likelihood of recombination between viral genes resulting in the production of virulent virus particles[90]. In order to evaluate the safety of replicons derived from V3014, newly formed replicons were tested by cytopathic effect (CPE) assay for the presence of any live VEE virus caused by recombination between replicon and helper plasmid genes. In the CPE assay, newly constructed V3014 VRPs are passaged two times in Vero cells, then inspected under a light microscope for CPE caused by live VEE virus. If no CPE is observed, then VRPs can be released from BL-3 containment and used in humans and animals. This assay has been approved by the Food and Drug Administration.

Once constructed, infectious titer of the VRPs was determined by indirect fluorescent antibody (IFA) assay. Serial dilutions of VRP were incubated with Vero cells for 30 to 60 minutes at 37ºC, 5%CO_2, and then cells were incubated for 18 to 20 hours at 37ºC, 5%CO_2. Following incubation, cells were washed, fixed, and stained with an antigen-specific primary antibody followed by a FITC-conjugated secondary antibody. Cells were observed using ultraviolet fluorescence microscopy (Nikon Eclipse TE300) and antigen-positive cells were counted to determine the titer of VRPs expressed as infectious units (IU)/mL.

Trial I Animals:

Twenty-four 3-week-old pigs arrived to ISU on Day -7 from a herd believed to be swine influenza virus (SIV) negative based on prior serology (Northwood, Randy Witt). Sow number and date of birth were recorded prior to the pigs being taken into the Biosafety Level-2 (BL2) Livestock Infectious Disease Isolation Facility (LIDIF) and weighed. Pigs were stratified by sow and weight and randomized using SAS software into 6 groups of 4 pigs per group. The three negative control (Gag) groups were housed in a room separate from the three HA vaccinate groups (see Table 1). Pigs were housed by group on elevated decks with no nose to nose contact with other groups within a room. Blood was collected 4 days prior to immunization (Day -4) and submitted to the Iowa State University Veterinary Diagnostic Lab for hemagglutination-inhibition (HI) testing using SIV H1N1 (99) and H3N2. Nasopharyngeal swabs were collected on Day 0 and tested for influenza antigen using the Directigen Flu A test kit (Becton Dickinson). The strain of human influenza used in the vaccine (A/Wyoming/03/2003) was not available pre-immunization for HI assay and thus pre-bleed serum was archived to be tested at a later date.

Trial I Immunizations:

Virus-like replicon particles (VRPs) derived from Venezuelan equine encephalitis virus were sent from AlphaVax Inc. (Research Triangle Park, North Carolina) to ISU on dry ice and immediately placed at -80ºC until used. V3014 HA-VRP expressed the hemagglutinin (HA) gene from human influenza virus A/Wyoming/03/2003 (H3N2). V3014 Gag-VRP expressed the gag gene from HIV and was used as a negative control. Vaccine doses were prepared by diluting the stock VRP in filter sterilized PBS (pH 7.2, without Mg^{2+} and Ca^{2+}, Sigma, St. Louis, MO) with 1% swine serum (obtained from pig #6 on Day -4 of this study). Formulations were held on ice and administered intramuscularly to pigs for immunizations 1

and 2 and either intramuscularly (2 of 4 pigs per group) or intradermally (2 of 4 pigs per group) for immunization 3 to the pigs within 1 hour.

The doses of VRPs given are shown in Table 1. Pigs were immunized on Days 0, 28, and 57. Groups received either 10^5, 10^6, or 10^7 infectious units (IU) for the first and second immunizations. However, all pigs received 10^8 IU of the appropriate VRP on Day 57. Following the first immunization, blood was collected on days 7, 14, 21, 28, 35, 42, 56, 64, and 77. Blood was centrifuged for 25 minutes at 1,200 x g, 4ºC to collect serum. Serum was frozen at -20ºC for storage.

Trial I Necropsy:

Pigs were euthanized on Day 77 via electrocution in accordance with AVMA suggested guidelines. Blood was collected in SST Plus blood collection tubes, and injection sites inspected for lesions. Serum was collected as described above and frozen at -20ºC for storage.

Table 1: Experimental design for Trial I.

Group # (4 pigs/group)	**VRP Treatment**	**Route[a]**	**Dose on Days 0 and 28 (IU)**	**Dose on Day 57 (IU)**
1	V3014 HA	IM/ID	10^5	10^8
2	V3014 HA	IM/ID	10^6	10^8
3	V3014 HA	IM/ID	10^7	10^8
4	V3014 Gag	IM/ID	10^5	10^8
5	V3014 Gag	IM/ID	10^6	10^8
6	V3014 Gag	IM/ID	10^7	10^8

[a] All pigs were vaccinated intramuscularly (IM) in the ham on Days 0 and 28. 2 of 4 pigs in each group were vaccinated intradermally (ID), and the other 2 were vaccinated intramuscularly on Day 57.

Trial II Animals:

Thirty 3-week-old pigs arrived on Day -7 from a different herd than used in Trial I (Ledger Swine Farms, Gary Ledger) in an effort to find pigs that were free of maternal antibody against A/Wyoming/03/2003. Pigs were weighed and then randomized using Research

Randomizer software into 6 groups of 5 pigs per group. Groups 1, 2, and 6 were housed in one room and groups 3, 4 and 5 in another (see Table 2). Pigs in each pen had no nose to nose contact with pigs in the other two pens. Blood was collected 7 days prior to immunization (Day -7) and tested by hemagglutination-inhibition assay for antibodies against A/Wyoming/03/2003.

Trial II Immunizations:

VRPs were obtained from the same source as in Trial I (AlphaVax Inc.). Gag-VRP expressed the Gag gene from HIV and was used as a negative control, as in Trial I. V3014 HA-VRP and TC-83 HA-VRP both expressed the hemagglutinin (HA) gene from human influenza virus A/Wyoming/03/2003.

Vaccine doses were prepared by diluting the stock VRP in filter sterilized PBS (pH 7.2, without Mg^{2+} and Ca^{2+}, Sigma, St. Louis, MO) with 1% swine serum (obtained from pig #29 on Day -7 of this study) and formulations were held on ice and administered to pigs within 1 hour. The doses of VRPs given are shown in Table 2. Pigs were immunized on Days 0 and 28. Nine of 30 pigs were also immunized again on Day 53 (see Table 2). Groups received 10^9 IU for all immunizations. Following the first immunization, blood was collected in SST Plus blood collection tubes (Becton Dickinson, Franklin Lakes, NJ) on days 14, 28, 42, and 52. Blood was centrifuged for 25 minutes at 1,200 x g, 4ºC to collect serum. Serum was frozen at -20ºC for storage.

Trial II Necropsy:

On Day 63, pigs were euthanized using intravenous Sleepaway (Fort Dodge Animal Health, Fort Dodge, IA) and weighed, followed by exsanguination. Blood was collected in SST Plus blood collection tubes. Serum was collected as stated above and frozen at -20ºC for storage.

Table 2: Experimental design for Trial II.

Group # (5 pigs/group)	VRP Treatment	Route[a]	Dose on Days 0 and 28 (IU)	Dose on Day 57 IU)
1	TC-83 HA	IM	10^9	none
2	TC-83 HA	IN/IM	10^9	$*10^9$
3	V3014 HA	IM	10^9	none
4	V3014 HA	IN/IM	10^9	$*10^9$
5	V3014 Gag	IM	10^9	none
6	V3014 Gag	IN/IM	10^9	$*10^9$

[a] Pigs were vaccinated either intramuscularly (IM) in the ham or intranasally (IN).
* Three of 5 pigs in each of groups 2, 4, and 6 were vaccinated intramuscularly on Day 57. The remaining pigs were not vaccinated after Day 28.

Virus Propagation for Hemagglutination Inhibition Assay:

Influenza virus A/Wyoming/03/2003 (H3N2) was obtained from Dr. Sasha Alexander (Centers for Disease Control, Atlanta, GA). Embryonated chicken eggs, approximately 11 days old, were purchased from the National Veterinary Services Laboratories (Ames, Iowa). Eggs were candled and marked along the air sac. An engraver was used to make a hole over the air sac of the egg, just large enough to insert a tuberculin syringe needle into the egg. A tuberculin syringe was used to inject 100µL of A/Wyoming virus diluted to 10^3 infectious particles/mL into the chorioallantoic cavity of the egg. The eggs were sealed and incubated at 37ºC without CO_2. Embryos were checked daily, and any that died within 24 hours were discarded. After 2-3 days, eggs were chilled for at least 4 hours at 4ºC, then virus was aseptically harvested by opening the shell over the air sac and pipetting out the clear allantoic fluid containing the virus. The fluid was pooled and centrifuged for 15 minutes at 750 x g, 4ºC. Supernatant containing the virus was collected and stored at -80ºC.

Hemagglutination-Inhibition Assay:

The hemagglutination inhibition (HI) assay was performed as previously described[83]. Briefly, turkey erythrocytes diluted 1:1 in Alsever's solution were purchased from the National Veterinary Services Laboratories (Ames, Iowa). Upon arrival, turkey erythrocytes were washed three times in sterile PBS (pH 7.4) by adding approximately 30 mL PBS to 15

mL erythrocyte solution, gently inverting to mix, then centrifuging for 10 minutes at ~370 x g, 4ºC. After the third wash, a 0.4% erythrocyte solution was made and stored for a maximum of seven days at 4ºC for use in the HI assay. The remaining erythrocyte pellet was stored for a maximum of seven days at 4ºC for use in the treatment of serum.

Serum collected from pigs was diluted 1:3 in receptor destroying enzyme (DENKA, Seiken) and allowed to incubate for 18-20 hours at 37ºC. Serum samples were then heat inactivated for 30 minutes at 56ºC, allowed to cool to room temperature, and then diluted to a final concentration of 1:9 in sterile PBS (pH 7.4). Washed, packed turkey erythrocytes were added to each sample at a concentration of 1:20. Serum plus erythrocytes were mixed by pipetting, and then allowed to incubate at room temperature for 30 minutes. The serum mixture was then centrifuged for five minutes at approximately 12,000 x g on a table top centrifuge. Serum was pipetted off of the erythrocyte pellet into clean microcentrifuge tubes and serum samples were stored at 4ºC for use within the next 5 days, or placed at -20ºC for extended storage.

Hemagglutination was performed using 0.4% turkey erythrocytes and four hemagglutinating units of influenza virus A/Wyoming/03/2003. A titer $\geq$ 1:40 was considered positive.

Hemagglutinin ELISA:

The hemagglutinin (HA) ELISA was preformed at AlphaVax, Inc. (Research Triangle Park, NC). Serum samples collected from pigs were sent on ice to AlphaVax. To measure antigen-specific humoral immune responses to HA in animals immunized with the HA-VRP vaccine, purified recombinant HA antigen derived from A/Wyoming/03/2003 (Protein Sciences Inc) was used in an ELISA. Briefly, 96-well Maxisorp ELISA plates (Nunc, Naperville, IL) were coated with 50 ng/well of recombinant HA protein in carbonate buffer. After overnight incubation at 4ºC, unbound antigen was discarded and each well was

incubated for 1 hr with blocking buffer (PBS containing 3% bovine serum albumin) at room temperature. After washing 6 times with PBS, test sera that were serially 2-fold diluted in diluent buffer (PBS with 1% BSA and 0.05% Tween-20) were added in triplicate to antigen-coated wells. Antigen-coated wells that received no serum served as background controls. Plates were incubated for 1 hour at room temperature, and then rinsed six times with PBS. Alkaline phosphatase-conjugated anti-pig IgG (whole molecule) antibody (Sigma, St. Louis, MO) was added to each well and incubated for 1 hr at room temperature. Wells were rinsed again six times with PBS before addition of alkaline phosphatase substrate and chromogen. Samples were read at 405 nm on a PowerWave 200 Microplate Scanning Spectrophotometer (BioTek Instruments, Winooski, VT). Endpoint titers were calculated using SOFTmax (Molecular Device Corp., Menlo Park, CA), and mean antibody titers were determined for each group. Titers are reported as the reciprocal of the serum dilution at which the A_{405} is ≥ 0.2.

Virus-like Replicon Particle Neutralization Assay:

The virus-like replicon particle (VRP) neutralization assay was preformed at AlphaVax, Inc. (Research Triangle Park, NC) to measure neutralizing antibody responses in the pigs against the VRPs. Serum samples were heat inactivated by incubation at 56°C for 30 minutes. Each serum sample was diluted 1:10 in EMEM. The diluted serum samples were then further serially diluted in 2 fold increments. VRP expressing green fluorescent protein (GFP-VRP) were added to each dilution and incubated at 4°C overnight.

Vero cells grown in 96 well tissue culture plates (~4 x 10^4 / cells well) were incubated for 16 – 18 hours, then media was removed from each well and 50µl of the serum/VRP samples was transferred to each well of the 96 well plate. The serum/VRP was incubated on the cells for 1 hr at 37°C, the samples were removed from the cells and 0.1mL of fresh media/well replaced. The plates were incubated overnight at 37°C. After overnight

incubation the media was replaced with 0.1mL of PBS. The reduction in GFP positive cells (compared to control serum treated VRP) was analyzed using ultraviolet fluorescence microscopy (Nikon Eclipse TE300). The VRP neutralizing antibody titer was expressed as the reciprocal end point dilution at which 80% of the GFP-VRPs were neutralized.

Statistics:

Tukey-Kramer HSD analysis was used to compare HI titers of pigs by group for each day that blood was collected. In order to allow for log transformation of titers with a value of 0, an arbitrary value of 0.5 was added to all HI titers, and then titers were $\log_{10}$ transformed, and analysis was preformed on log transformed data. Analysis was conducted using the statistical software JMP 6.0.0 (SAS Institute, Cary, NC).

Results

Trial I:

All 24 pigs in Trial I tested negative by hemagglutination-inhibition (HI) assay for antibody against SIV H3N2 on Day -4 (data not shown). One pig of 24 (pig 16) was suspect positive by HI for antibody against SIV H1N1 (99) with a titer of 40 (data not shown). The other 23 pigs tested negative for antibody against SIV H1N1 (99). All 24 pigs tested negative for the presence of influenza A antigen by the Directigen Flu A test kit (Becton Dickinson) (data not shown).

In Trial I, 23 of 24 pigs had a maternally derived antibody (MDA) HI titer of 20 or greater against influenza A/Wyoming/03/2003 on Day -4, while one pig (pig 24) had a maternal antibody titer of 0 by HI assay on Day -4 (see Table 3). Geometric mean titers were determined for each group (see Figure 1). There was no significant difference in HI titers between any of the groups on any of the days. Pig 24 did appear to respond to vaccination, and had an HI titer of 320 by Day 64 of the trial (data not shown), but data from one pig does

not provide enough evidence to make any specific conclusions about the ability of VRPs to elicit any immune response in pigs against HA. The data shown from Trial I demonstrated the need to find a swine herd that did not have antibodies to influenza A/Wyoming/03/2003.

Table 3: Trial 1- Hemagglutinin-Inhibition antibody titer of pigs on Day -4.

Group #	**Pig #**	**Sow #[a]**	**HI titer Day -4[b]**	**Group #**	**Pig #**	**Sow #[a]**	**HI titer Day -4[b]**
1	6	24	20	4	1	24	20
V3014	10	97551	80	V3014	8	24	20
HA	14	91	40	Gag	11	97551	80
10^5	22	93	80	10^5	18	91	80
2	3	24	20	5	5	24	20
V3014	4	24	20	V3014	7	24	20
HA	13	97551	160	Gag	17	91	80
10^6	20	93	80	10^6	21	93	80
3	9	24	20	6	2	24	20
V3014	12	97551	160	V3014	15	91	80
HA	16	91	80	Gag	19	91	80
10^7	24	93	0	10^7	23	93	80

[a] Pigs from Trial I came from 4 different sows (sow 24, 91, 93, and 97551)
[b] Titers represent maternally derived antibody. Pig 24 was the only pig without maternally derived antibody on Day -4

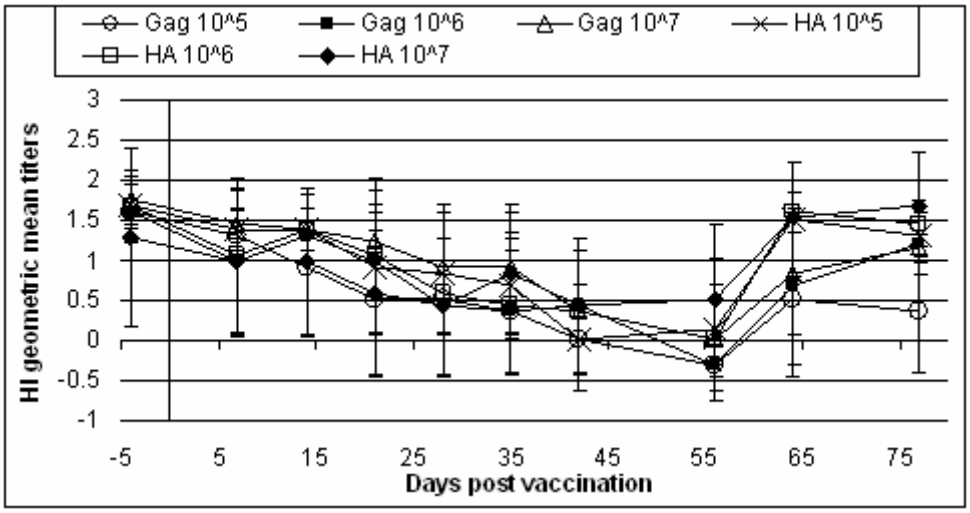

Figure 1: Trial I - Hemagglutination-Inhibition geometric mean titers (GMT) of pigs by group. Group 1: HA 10^5, Group 2: HA 10^6, Group 3: HA 10^7, Group 4: Gag 10^5, Group 5: Gag 10^6, Group 6: Gag 10^7. GMT were calculated by taking the $\log_{10}$(HI titers + 0.5) of pigs on all days, then calculating the mean of the logs. Error bars represent 95% confidence interval.

Trial II:

In Trial II, 9 of 30 pigs had an initial MDA HI titer of 160 or greater, while the other 21 pigs tested negative (titers = 0) for MDA against A/Wyoming by HI assay (see Table 4). Figure 2 shows the geometric mean titers of pigs by group. On Days -7, 14, 28, 42, and 52 there was no significant difference in HI titers between any of the groups. On Day 63, pigs vaccinated IM with either TC-83 HA or V3014 HA (Groups 1 and 3) had significantly higher HI antibody titers than pigs vaccinated IN with either TC-83 or V3014 (Groups 2 and 4) and pigs vaccinated either IM or IN with 3014 Gag (negative controls, Groups 5 and 6). On day 63, there was no significant difference in HI titers between pigs in Group 1 versus Group 3, demonstrating that VRPs derived from either TC-83 or V3014 work equally well at eliciting an immune response against HA in pigs. On Day 63 there were no significant differences between Groups 2, 4, 5, and 6, demonstrating that the IN route of vaccination was less efficient than the IM route at eliciting a serum antibody response against HA in the pigs.

ELISA data is shown in Table 5 and Figures 3-6. Six of 20 pigs in groups vaccinated with HA-VRPs (groups 1-4) had MDA against A/Wyoming. Table 5 shows the OD_{405} values for pigs vaccinated with HA-VRPs on Days -7, 14, and 28. The 6 pigs with MDA had OD_{405} values > 0.5 on Day -7, while pigs without MDA had OD_{405} values < 0.1 on Day -7. By Day 14, the six pigs with MDA had lower OD_{405} values, showing decay in their maternal antibodies, but pigs without MDA started to show an increase in OD_{405} values, indicating a possible response to vaccination. By Day 28, OD_{405} values for pigs without MDA had increased further, with most pigs having an OD_{405} value > 0.1, therefore serum collected from pigs on Day 14 was used as baseline or "negative" serum to calculate ELISA titers on Days 42, 52, and 63. Titers of all pigs in groups 1-4 may be considered < 40 on Days -7, 14, and 28. HA ELISA was not run on serum from pigs vaccinated with Gag-VRPs (Groups 5 and 6).

Figures 3 and 4 show HA ELISA titers of pigs vaccinated either IN or IM with 10^9 IU of TC-83 HA-VRP. Figures 5 and 6 show HA ELISA titers of pigs vaccinated either IN or IM with 10^9 IU of V3014 HA-VRP. Pigs vaccinated IN (Figures 3 and 5) had lower ELISA titers than pigs vaccinated IM (Figures 4 and 6). Titers for pigs vaccinated IM with either TC-83 or V3014 HA-VRP (Figures 4 and 6) were comparable between these two groups.

Table 6 shows VRP neutralization titers for pigs in on Days -7, 14, and 28. Pigs in groups 1, 2, 4, and 6 had low or no levels of antibodies against the VRP vectors on all 3 days, while pigs in groups 3 and 5 had titers of ≤ 1280 by day 28. Pigs in group 3 were vaccinated intramuscularly in the ham with 10^9 IU of V3014 HA-VRP, while pigs in group 5 were vaccinated intramuscularly in the ham with 10^9 IU of Gag-VRP.

Table 4: Trial II - Hemagglutination-Inhibition titers on Day -7. Nine of 30 pigs were positive by HI for the presence of maternally derived antibody.

Group #	**Pig #**	**HI Titer Day -7**	**Group #**	**Pig #**	**HI Titer Day -7**
1	2	160	4	8	0
TC-83	9	320	V3014	12	0
IM	14	0	IN	17	160
	18	320		22	0
	29	0		26	0
2	4	0	5	3	320
TC-83	11	320	Gag	7	320
IN	20	0	(Negative	10	0
	23	0	Control)	16	0
	28	0	IM	27	0
3	1	0	6	15	0
V3014	5	160	Gag	19	0
IM	6	0	(Negative	24	320
	13	0	Control)	25	0
	21	0	IN	30	0

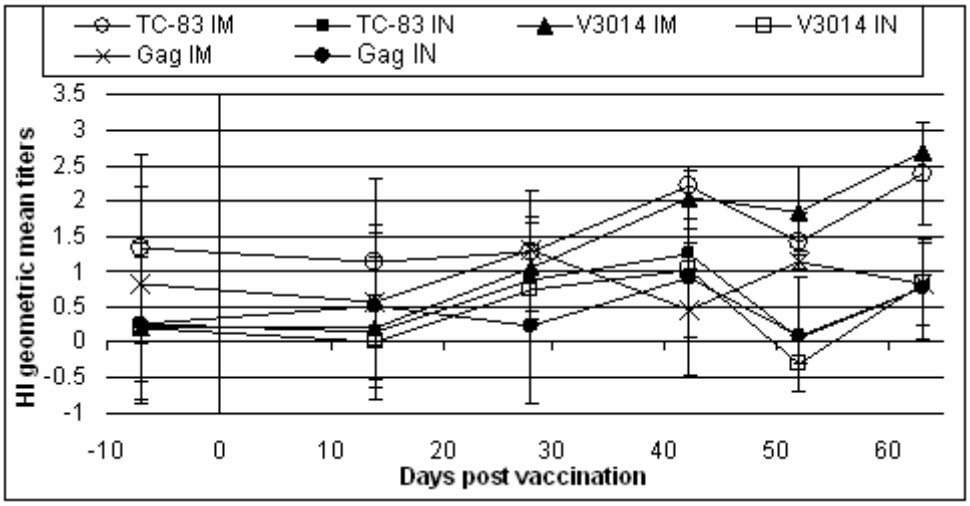

Figure 2: Trial II - Hemagglutination-Inhibition geometric mean titers (GMT) of pigs by group. Group 1: TC-83 IM, Group 2: TC-83 IN, Group 3: V3014 IM, Group 4: V3014 IN, Group 5: Gag IM, Group 6: Gag IN. GMT were calculated by taking the $\log_{10}$(HI titers + 0.5) of pigs on all days, then calculating the mean of the logs. Error bars represent 95% confidence interval.

Table 5: Trial 2 ELISA Optical Density values for Days -7, 14 and 28.

Group #	Pig #	ELISA OD_{405} at 1:40 dilution			Group #	Pig #	ELISA OD_{405} at 1:40 dilution		
		Day -7	Day 14	Day 28			Day -7	Day 14	Day 28
1	2*	1.14	0.462	0.324	3	1	0.094	0.307	0.460
	9*	1.27	0.707	0.485		5*	1.240	0.597	0.405
	14	0.082	0.163	0.267		6	0.087	0.105	0.187
	18*	1.20	0.653	0.448		13	0.092	0.111	0.159
	29	0.081	0.101	0.133		21	0.086	0.085	0.141
2	4	0.088	0.095	0.111	4	8	0.095	0.091	0.118
	11*	1.17	0.453	0.380		12	0.093	0.082	0.098
	20	0.095	0.080	0.095		17*	0.514	0.181	0.141
	23	0.090	0.080	0.109		22	0.086	0.094	0.102
	28	0.091	0.081	0.100		26	0.080	0.084	0.115

*indicates pigs with maternal antibody against A/Wyoming.

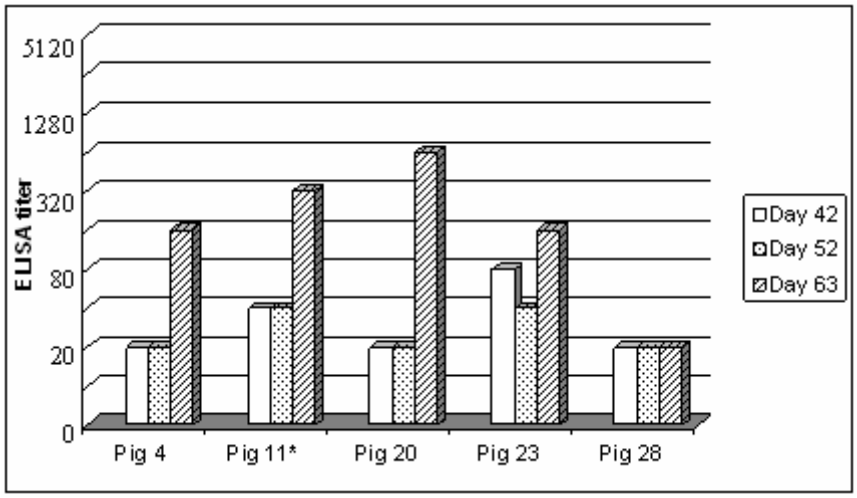

Figure 3: HA ELISA titers for Trial 2, group 2, pigs vaccinated intranasally with 10^9 IU of TC-83 HA-VRP on Days 0 and 28. Pigs 4, 11 and 20 were vaccinated for a third time intramuscularly in the ham on Day 53 with 10^9 IU of TC-83 HA-VRP. ELISA titers are defined as the reciprocal of the highest dilution of serum for which the optical density was ≥ 2 times the optical density of serum diluted 1:40 from Day 14. *denotes pig(s) with maternal antibody against A/Wyoming.

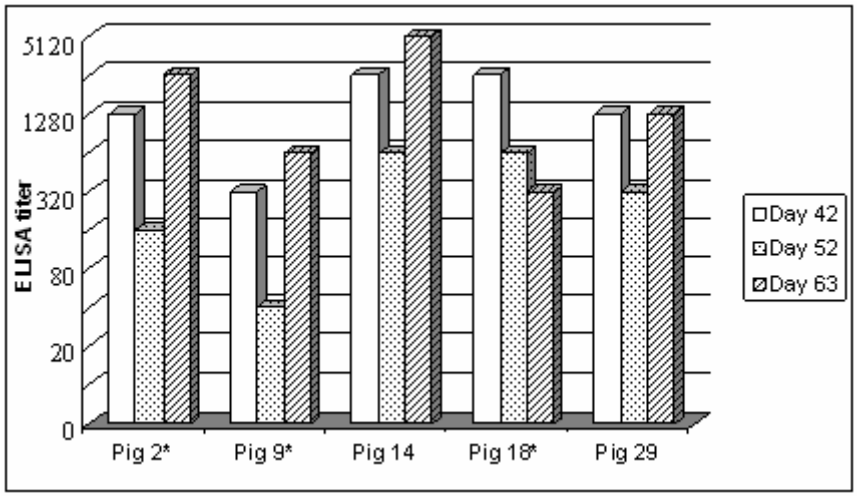

Figure 4: HA ELISA titers for Trial 2, group 1, pigs vaccinated intramuscularly in the ham with 10^9 IU of TC-83 HA-VRP on Days 0 and 28. No pigs in group 1 were vaccinated after Day 28. ELISA titers are defined as the reciprocal of the highest dilution of serum for which the optical density was ≥ 2 times the optical density of serum diluted 1:40 from Day 14. *denotes pig(s) with maternal antibody against A/Wyoming.

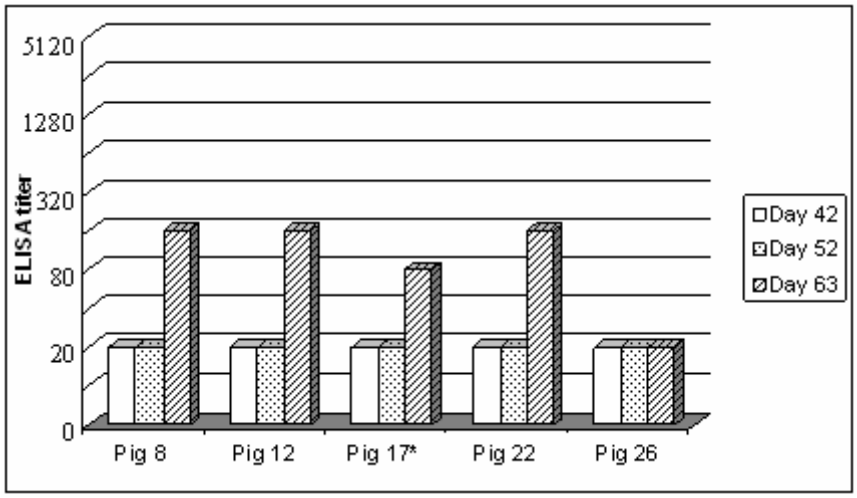

Figure 5: HA ELISA titers for Trial 2, group 4, pigs vaccinated intranasally with 10^9 IU of V3014 HA-VRP on Days 0 and 28. Pigs 8, 12 and 17 were vaccinated for a third time intramuscularly in the ham on Day 53 with 10^9 IU of V3014 HA-VRP. ELISA titers are defined as the reciprocal of the highest dilution of serum for which the optical density was ≥ 2 times the optical density of serum diluted 1:40 from Day 14. *denotes pig(s) with maternal antibody against A/Wyoming.

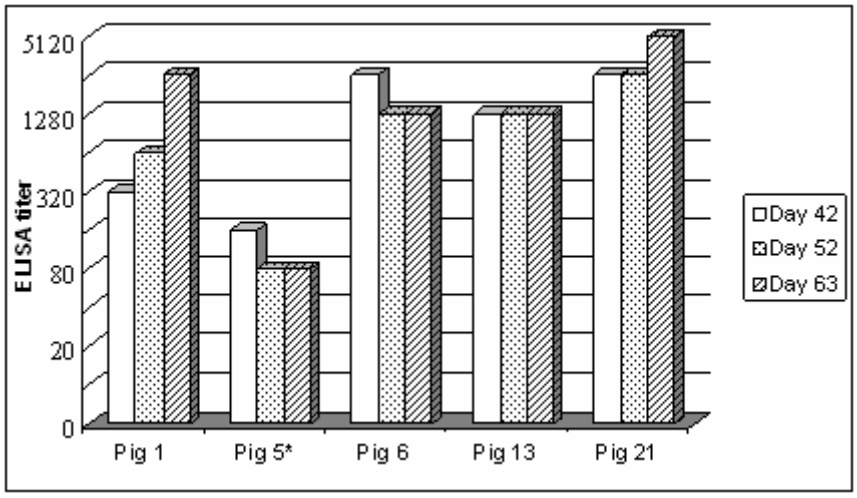

Figure 6: HA ELISA titers for Trial 2, group 3, pigs vaccinated intramuscularly in the ham with 10^9 IU of V3014 HA-VRP on Days 0 and 28. No pigs in group 3 were vaccinated after Day 28. ELISA titers are defined as the reciprocal of the highest dilution of serum for which the optical density was ≥ 2 times the optical density of serum diluted 1:40 from Day 14. *denotes pig(s) with maternal antibody against A/Wyoming.

Table 6: Trial 2 VRP neutralization data.

Group #	Pig #	VRP Neutralization Titer			Group #	Pig #	VRP Neutralization Titer		
		Day -7	Day 14	Day 28			Day -7	Day 14	Day 28
1	2	< 20	40	< 20	4	8	< 20	< 20	< 20
	9	< 20	20	< 20		12	< 20	< 20	< 20
	14	< 20	< 20	< 20		17	< 20	< 20	< 20
	18	< 20	40	< 20		22	< 20	< 20	< 20
	29	< 20	20	< 20		26	< 20	< 20	< 20
2	4	< 20	< 20	< 20	5	3	< 20	160	320
	11	< 20	20	< 20		7	< 20	160	160
	20	< 20	< 20	< 20		10	< 20	1280	1280
	23	< 20	< 20	< 20		16	< 20	40	320
	28	< 20	< 20	< 20		27	< 20	40	80
3	1	< 20	320	640	6	15	< 20	< 20	< 20
	5	< 20	>1280*	1280		19	< 20	< 20	< 20
	6	< 20	40	160		24	< 20	< 20	< 20
	13	< 20	160	640		25	< 20	< 20	-
	21	< 20	160	320		30	< 20	< 20	< 20

* value for pig 5 on Day 14 was not determined beyond 1280. Pig 25 was euthanized prior to Day 28 due to complications unrelated to the study.

Discussion

In Trial I our objective was to determine if VRPs expressing the hemagglutinin (HA) protein of influenza A/Wyoming/03/2003 would be able to elicit an immune response against the HA protein in pigs. Because VRPs had never been tested in pigs, concentrations of VRPs used as well as dosing schedule were based upon previous work using VRPs in other animal species including mice[11, 17, 54-56], guinea pigs[89], and nonhuman primates[18]. Due to the unexpected fact that only one pig of 24 did not have a MDA titer against A/Wyoming (pig 24) and this pig had the highest HI titer at necropsy (data not shown), further work was needed to determine if the response seen in this one pig was representative of a larger portion of the swine population. Also, serum from pig 6 on Day -4 was used in the VRP vaccine formulations at a concentration of 1%. Pig 6 had an HI antibody titer of 20 on Day -4 due to the presence of MDA, but this was not known until after the termination of the study. Due to the low concentration of serum in the vaccine formulation, it is not likely that this caused major changes in the HI titers of pigs in Trial I, but it is possible that the antibody against

influenza HA from this serum could have negatively impacted vaccination of pigs in this trial. Maternally derived antibodies have previously been shown to be inefficient at protecting piglets from experimental influenza infection against both homologous and heterologous virus strains[52, 61]. It has also been shown that MDA may interfere with the ability of some vaccines to elicit a protective immune response against influenza virus[52, 52].

In Trial II, our objectives were threefold. First, we wanted to determine if the results seen in Trial I for pig 24 (no maternal antibody against A/Wyoming) could be reproduced to demonstrate the capability of VRPs to elicit an immune response against influenza HA in pigs. Secondly, we wanted to determine if a difference could be detected in immune response to the HA protein between pigs vaccinated with VRPs derived from V3014 versus TC-83. Thirdly, we wanted to evaluate the difference between intramuscular (IM) versus intranasal (IN) routes of vaccination. Based upon hemagglutination-inhibition (HI) and ELISA titers, we demonstrated that VRPs derived from VEE are able to elicit an immune response in pigs. Based upon our data, the intramuscular (IM) route of vaccination appeared more effective at eliciting a serum antibody response in pigs against HA than the intranasal (IN) route when vaccinating pigs with VRPs. We also showed that VRPs derived from either TC-83 or V3014 were equally efficient at expressing the HA protein *in vivo*, as no significant difference was observed in immune responses between pigs vaccinated with either of these VRPs. Because V3014 is considered a select agent, VRPs derived from this VEE virus strain must be constructed under Biosafety-level 3 conditions. Replicons constructed from TC-83 may be constructed under Biosafety-level 2 conditions; therefore the use of TC-83 to construct VRPs is desirable in that production costs are less for VRPs derived from TC-83 than for VRPs derived from V3014.

Although the sample size of pigs is not large enough to make any specific conclusions, it is interesting to note that most pigs in Trial II with an initial MDA titer against

A/Wyoming and vaccinated either IM or IN with HA-VRPs maintained HI titers of ≥ 40 throughout most or all of the study (data not shown). Pigs in the negative control Gag-VRP groups that had an initial MDA titer against A/Wyoming all reached HI titers of ≤ 20 by Day 52. Work done with an adenovirus vaccine vector has shown promise as an effective vaccine against influenza in the presence of MDA. It was shown that piglets with MDA against influenza H3N2 could be protected against homologous challenge after vaccination with recombinant Ad5 vector expressing influenza HA and NP and boosted 3 weeks later with a commercial vaccine (End-FLUence 2)[135]. It is possible that VRP vaccines may be able to provide similar protection in the presence of MDA. Further work is needed to optimize vaccination dosing and schedule of VRP administration in order to determine the maximum response that can be achieved in pigs vaccinated with VRPs. More work is also needed to determine whether or not VRPs can elicit an immune response to HA in the presence of MDA, and whether or not the immune response elicited by VRPs is protective against virus challenge.

One problem faced by some viral vector vaccines is that the host develops an immune response against the vector itself, reducing the effectiveness of the vector to express its gene of interest. An example of this is human adenovirus vector vaccines, in which the development of a strong neutralizing antibody titer against adenovirus proteins has been shown to prevent the vector from effectively expressing protein after a single administration[111]. In Trial I, all pigs had a VRP neutralization titer of ≥ 20 by Day 64 (data not shown). Pig 24 had a VRP neutralization titer of >1280, yet still responded to VRP with an HI titer of 320 by necropsy (Day 77). In Trial II, only pigs in groups 3 and 5 had VRP neutralization titers of ≥ 20 by Day 28. More work is needed to determine whether VRP neutralization titers would prevent the use of another VRP vaccine from efficiently expressing its gene of interest in pigs. One way to circumvent this potential problem is to simultaneously vaccinate pigs with multiple VRPs each expressing one gene of interest or

one VRP expressing several different genes of interest to protect against a broad range of pathogens. Work done in mice has shown that replicons derived from Venezuelan equine encephalitis virus expressing genes from either Marburg virus, *Bacillus anthracis*, or *Clostridium botulinum* administered simultaneously were able to provide protection against challenge with *Clostridium botulinum*, demonstrating that VRPs expressing different proteins administered simultaneously do not inhibit the effectiveness of one another[55]. Further work is needed to determine if VRPs administered simultaneously can protect against challenge with multiple infectious agents.

CHAPTER 4. IMMUNOGENECITY IN PIGS OF INFLUENZA HEMAGGLUTININ PROTEIN DERIVED FROM AN ALPHAVIRUS REPLICON

Introduction

Improved methods of vaccination against influenza A virus in swine are needed to prevent economic loss to the swine industry due to increased length of time needed for pigs to reach market weight[80]. Influenza virus hemagglutinin (HA) is one of two major viral surface proteins, and antibody against the HA protein has been shown to be protective against virus challenge[93, 113]. Recently, recombinant protein technology has allowed for the development and mass production of recombinant HA (rHA) protein for use in vaccines against influenza virus[76]. Work done in mice has shown that rHA and neuraminidase (NA) proteins produced via baculovirus vectors were able to elicit a protective immune response against influenza challenge[45] and work done in chickens demonstrated that rHA protein vaccine was able to protect chickens from challenge with H5 and H7 influenza subtypes[15]. In clinical trials, the use of rHA produced via baculovirus expression vectors in insect cell lines has shown much promise for commercialization as a vaccine against influenza infection[66].

We wanted to evaluate the ability of rHA protein produced *in vitro* in Vero cells from alphavirus virus-like replicon particles (VRPs) to elicit an immune response in pigs. One trial was conducted in which rHA protein was used to vaccinate pigs intramuscularly either alone or with an adjuvant. Two different adjuvants were tested, Emulsigen-D (MVP Laboratories, Omaha, NE) and VRPs derived from Venezuelan equine encephalitis (VEE) virus. Previous work in mice demonstrated that when VRPs expressing an irrelevant gene (green fluorescent protein) were administered with an inactivated influenza virus, they had the ability to elicit a strong systemic and mucosal response to the influenza HA protein, even when administered at a non-mucosal site[120]. Therefore, these two adjuvants were each

evaluated for their ability to help stimulate a strong immune response against the HA protein in pigs.

Recombinant HA was produced from VRPs derived from Venezuelan equine encephalitis virus. We also challenged pigs with live A/Wyoming/03/2003 influenza virus in order to determine if an immune response elicited by replicon-derived HA protein would protect pigs from virus challenge.

Materials and Methods

Vaccine Preparation - HA and PRRS Protein Lysate:

The protocol for preparation of HA and PRRS lysate was obtained from AlphaVax Inc. (Research Triangle Park, NC). African green monkey kidney (Vero) cells obtained from American Type Culture Collection were propagated in growth media (DMEM supplemented with 4% fetal bovine serum and 0.2% gentamicin). Cells were allowed to grow for approximately 18 - 20 hours in 75 cm^2 cell culture flasks, and then cells in one flask were counted using a hemocytometer. First, growth media was removed and cells were rinsed with 2-3mL trypsin. Cells were then incubated with 5mL trypsin for approximately 5-10 minutes at 37ºC until cells detached from flask. Total volume of the cell solution was measured, then 100µL of cells was mixed with 400µL of Trypan Blue stain. Cells were pipetted into a hemocytometer, and cells were counted under a light microscope. Total cells per mL were determined according to the following equation:

Total cells/mL = (total cell count/ 5) x (1/ dilution of cells in Trypan Blue) x 10^4

Total number of cells per flask was then calculated. VRPs expressing either the hemagglutinin (HA) protein from A/Wyoming/03/2003 or the glycoprotein 5 (gp5) from porcine respiratory and reproductive syndrome (PRRS) virus (used as negative control

protein) were then diluted appropriately in growth media to be used to infect Vero cells at a multiplicity of infection (MOI) of 5. VRPs in growth media were added to cells and allowed to incubate for 1 hour at 37ºC, 5% CO_2. Flasks containing VRP infected cells were gently swirled every 15 minutes. After 1 hour, VRPs were pipetted off of the cells, and 40 mL growth media was added to each flask. Cells were incubated for 18 hours at 37ºC, 5% CO_2. After 18 hours, growth media was removed from the flasks, cells were washed one time with sterile PBS, pH 7.2, then 25mL of lysis buffer (10mM Tris-HCl pH 7.4, 1mM EDTA, 0.25 mM NaCl, 1% Triton X-100, and 1tablet/ 50mL Complete EDTA-Free Protease Inhibitor Cocktail, Roche, Basel, Switzerland) was added to each 75 cm^2 cell culture flask. Flasks were gently rocked for 7 – 10 minutes at room temperature to lyse cells. Cellular suspension was then pipetted from each flask and centrifuged for 25 minutes at ~4,500 x g, 4ºC. Supernatant was collected and tested via western blot for the presence of the desired protein. Supernatant was then used to vaccinate pigs in Trial 3, above, on Days 21 and 49.

Animals:

Thirty 3-week-old pigs arrived at ISU on Day -7 from a herd believed to be negative for influenza A/Wyoming/03/2003 (Ledger Swine Farms, Gary Ledger). Sow number was recorded. Pigs were randomized using Research Randomizer software into 6 groups of 5 pigs per group. Pigs were taken into the BL2 Livestock Infectious Disease Isolation Facility (LIDIF) and weighed. Pigs were housed in three separate pens in the same room. Groups 1 and 2 shared a pen, groups 3 and 4 shared the second pen, and groups 5 and 6 shared the third pen (see Table 1). Pigs in each pen had no nose to nose contact with pigs in the other two pens. Blood was collected 3 days prior to immunization (Day -3) and tested by hemagglutination-inhibition assay for antibodies against A/Wyoming/03/2003.

Immunizations:

For vaccine treatment administered on Day 0, VRP lysate was sent from AlphaVax, Inc. (Research Triangle Park, North Carolina). For vaccine treatment administered on Days 21 and 49, VRP lysate was prepared at Iowa State University. Samples of lysate used for all treatments were run in western blot to confirm the presence of influenza HA protein (see Figure 11).

Formulations were held on ice and administered to pigs within 1 hour. The doses of VRPs given are shown in Table 1. Pigs were immunized on Days 0 and 21, and 49 (see Table 1). Groups received $\leq 25\mu g$ protein/dose for all immunizations. A total volume of 1.25mL was given to each pig per dose for all immunizations. Sterile PBS (pH 7.2, without Mg^{2+} and Ca^{2+}, Sigma, St. Louis, MO) was added to all vaccine formulations to achieve a total volume of 1.25mL. For pigs vaccinated with HA or PRRS protein plus MVP Emulsigen-D adjuvant, protein lysate was added to the adjuvant at a ratio of 80:20, for a total volume of 1.25mL per dose. For pigs vaccinated with HA or PRRS protein plus VRP adjuvant, 10^8 infectious units (IU) of VRP were added to each vaccine dose, for a total volume of 1.25mL per dose. Following the first immunization, blood was collected on days 14, 28, 35, 48, 56, and 68.

Virus challenge:

On Day 63, pigs weighing approximately 100-120 lbs were held upright, and a 16 inch catheter tube (Davol, Cranston, RI) was inserted into the pigs' tracheas. Virus previously prepared in MDCK cells was used for challenge. Ten milliliters of virus at a concentration of 10^6 $TCID^{50}$/mL was injected into each pig's lungs through the catheter tube. Nasal swabs and rectal temperatures were recorded for the following four days post challenge. Nasal swabs were collected in sterile PBS supplemented with 0.2% gentamicin (100mg/mL) and 2% penicillin-streptomycin solution (10,000units/mL).

Necropsy:

On Day 68, pigs were euthanized using intravenous Sleepaway and weighed, followed by exsanguination. Blood was collected in SST Plus blood collection tubes. Serum was collected as stated above and frozen at -20ºC for storage. Lungs were removed and inspected for influenza lesions. No apparent lesions were present. Bronchoalveolar lavage fluid was collected by pipetting 25 mL of lavage fluid (MEM media supplemented with 0.1% gentamicin (100mg/mL) and 1% penicillin-streptomycin solution (10,000units/mL)) into each set of lungs, gently massaging lungs, and collecting approximately 3-12 mL lavage fluid. Lavage fluid was stored at -80ºC. Samples of lung tissue from each pig were collected in formalin for histopathology and immunohistochemistry and in sterile whirlpak bags for virus isolation or PCR. Lung tissue in bags was stored at -80ºC.

Table 1: Experimental design for Trial I.

Group # (5 pigs/group)	**Subunit Treatment**	**Route[a]**	**Treatment Dose on Days 0, 21 and 49 (µg protein/dose)[b]**	**Virus Challenge Dose on Day 63 (10^6 $TCID^{50}$ /mL)**
1	PRRS lysate	IM	≤ 25	10 mL, intratracheal
2	V3014 HA lysate	IM	≤ 25	10 mL, intratracheal
3	PRRS lysate + MVP adjuvant	IM	≤ 25	10 mL, intratracheal
4	V3014 HA lysate + MVP adjuvant	IM	≤ 25	10 mL, intratracheal
5	PRRS lysate + V3014 HA VRP	IM	≤ 25	10 mL, intratracheal
6	V3014 HA lysate + PRRS VRP	IM	≤ 25	10 mL, intratracheal

[a] Pigs were vaccinated intramuscularly (IM) in the ham.

[b] HA protein concentration of the HA lysate for the dose given on Day 0 was determined to be 25 µg/dose of 0.714 mL. The PRRS protein concentration and the HA protein concentration for Days 21 and 49 were not determined, but thought to be ≤ 25 µg/dose.

Preparation of Challenge Virus:

Madin Darby canine kidney (MDCK) cells (passage 63) were obtained from Dr. Pravina Kitikoon, Thacker Lab, Iowa State University, Ames, Iowa. Cells were originally received from the American Type Culture Collection. Cells were propagated in growth media consisting of 1x MEM supplemented with 6% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin, and 0.1% gentamicin. When cells reached approximately 95% confluency, the monolayer was washed twice with sterile phosphate buffered saline (PBS) pH 7.2. Infecting media consisting of 1x MEM supplemented with 6% bovine serum albumin (BSA), 1% L-glutamine, 1% penicillin-streptomycin, 0.1% gentamicin and 1μg/mL of TPCK treated trypsin was added to cells. Virus was then added to cells at a concentration of 10^5 to 10^6 infectious particles per flask in a volume of no less than 100μL per flask. Cells were incubated at 37ºC, 5% CO_2.

When 50% to 70% of virally infected cells had lifted off of the flask, virus was harvested. First, flasks of virally infected cells were placed at -80ºC until the media was completely frozen. Flasks were then removed from the freezer and allowed to thaw at room temperature. Cell debris was clarified by centrifuging the cell media for 15 minutes at ~2,000 x g, 4ºC. Supernatant containing virus was stored at -80ºC.

Virus Isolation/Titration:

Virus was isolated and viral titrations performed as previously described[52]. Briefly, MDCK cells were propagated in 96-well cell culture plates as described above, with 100mL of media per well. Samples (either bronchoalveolar lavage fluid, nasal swab fluid, or virus previously grown in eggs or MDCK cells) containing unknown amounts of virus were serially diluted 10-fold in infecting media, and 100μL of each dilution was plated into each well. Positive and negative controls were included on each plate. Plates were incubated at 37ºC, 5% CO_2 for 36 to 40 hours. Plates were then stained using a protocol as previously described[52].

Western Blot:

All western blots were run under non-reducing conditions using 12 well, 4%–12% NuPAGE Novex Bis-Tris Gels (Invitrogen, Carlsbad, CA) in a XCell Sure Lock Electrophoresis Cell (Invitrogen, Carlsbad, CA). Serum or bronchoalveolar lavage fluid from pigs was tested for the presence of antibody against the hemagglutinin protein of influenza A/Wyoming/03/2003. Whole influenza A/Wyoming/03/2003 virion particles were used as protein samples, and serum or bronchoalveolar lavage (BAL) fluid was used as primary antibody in each western blot.

Samples were prepared by mixing 15µL of Influenza A/Wyoming/03/2003 with 7.5µL of NuPAGE LDS sample buffer 4x (Invitrogen, Carlsbad, CA) and 7.5µL of deionized water for each sample. Samples were then placed in boiling water for 5 minutes. 1x NuPAGE MES SDS buffer was prepared according to manufacturer's instructions (Invitrogen, Carlsbad, CA). 10µL of SeeBLUE Plus 2 Ladder (1x) (Invitrogen, Carlsbad, CA) was run in each gel. 25µL of sample was then added to each respective well. Influenza A/Wyoming/03/2003 was used as the positive control, and swine serum previously shown to contain no antibodies against A/Wyoming/03/2003 was used as the negative control. Samples were run at 200V constant, for 50 minutes.

Transfer pads and blotting filter paper were soaked for 30 minutes in transfer buffer (0.025M BisTris, 0.025M Bicine, 0.001M EDTA disodium salt, and 10% methanol in filtered, deionized water). PVDF membrane (Millipore, Billerica, MA) was soaked in 100% methanol for 15 seconds, and then placed in transfer buffer for 30 minutes.

When samples had completed running in the gel, the transfer pads, filter paper, PVDF membrane and gel, were placed in the transfer apparatus according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA), the inner chamber of the blot apparatus was filled with transfer buffer, and the outer chamber was filled with deionized water. The transfer was performed at 170mA constant, for 75 minutes.

Immediately following transfer, SeeBLUE ladders were separated from the PVDF membrane, then the membrane was cut into strips and placed in blocking buffer (wash buffer containing 5% non-fat evaporated milk) on a rocker for 1 hour at room temperature. Following blocking, each strip was placed in primary antibody solution (1:100 dilution of serum or BAL in blocking buffer) on a rocker for 18-24 hours at 4ºC.

Primary antibody was removed and each strip was washed 3 times with wash buffer (0.0015M KH_2PO_4, 0.02M Na_2HPO_4 dibasic anhydrous, 0.134M NaCl, 0.0027M KCl, and 0.05% Tween-20 in filtered, deionized water) for 10 minutes on a rocker. Strips were then placed in secondary antibody solution (1:2000 dilution of horse-radish peroxidase labeled α-swine immunoglobulin (will detect all swine immunoglobulins) in blocking buffer) on a rocker for 1 hour at room temperature. Secondary antibody was removed and each strip was washed 3 times with wash buffer for ten minutes on a rocker.

Proteins were analyzed using KPL peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MA) according to manufacturer's instructions. Strips were soaked in the substrate solution for approximately 3 minutes for serum samples and 12 minutes for BAL samples, then immediately placed in deionized water. Strips were allowed to dry, then the membrane was reassembled and protein bands were analyzed.

Virus Propagation for Hemagglutination Inhibition Assay:

Influenza virus A/Wyoming/03/2003 (H3N2) was obtained from the Centers for Disease Control (Atlanta, GA). Embryonated chicken eggs, approximately 11 days old, were purchased from the National Veterinary Services Laboratories (Ames, Iowa). Eggs were candled and marked along the air sac. An engraver was used to make a hole over the air sac of the egg, just large enough to insert a tuberculin syringe needle into the egg. A tuberculin syringe was used to inject 100µL of A/Wyoming virus diluted to 10^3 infectious particles/mL into the chorioallantoic cavity of the egg. The eggs were sealed and incubated at 37ºC

without CO_2. Embryos were checked daily, and any that died within 24 hours were discarded. After 2-3 days, eggs were chilled for at least 4 hours at 4ºC, then virus was aseptically harvested by opening the shell over the air sac and pipetting out the clear allantoic fluid containing the virus. The fluid was pooled and centrifuged for 15 minutes at 750 x g, 4ºC. Supernatant containing the virus was collected and stored at -80ºC.

Hemagglutination-Inhibition Assay:

The hemagglutination inhibition (HI) assay was performed as previously described[83]. Briefly, turkey erythrocytes diluted 1:1 in Alsever's solution were purchased from the National Veterinary Services Laboratories (Ames, Iowa). Upon arrival, turkey erythrocytes were washed three times in sterile PBS (pH 7.4) by adding approximately 30 mL PBS to 15 mL erythrocyte solution, gently inverting to mix, then centrifuging for 10 minutes at ~370 x g, 4ºC. After the third wash, a 0.4% erythrocyte solution was made and stored for a maximum of seven days at 4ºC for use in the HI assay. The remaining erythrocyte pellet was stored for a maximum of seven days at 4ºC for use in the treatment of serum.

Serum collected from pigs was diluted 1:3 in receptor destroying enzyme (DENKA, Seiken) and allowed to incubate for 18-20 hours at 37ºC. Serum samples were then heat inactivated for 30 minutes at 56ºC, allowed to cool to room temperature, and then diluted to a final concentration of 1:9 in sterile PBS (pH 7.4). Washed, packed turkey erythrocytes were added to each sample at a concentration of 1:20. Serum plus erythrocytes were mixed by pipetting, then allowed to incubate at room temperature for 30 minutes. The serum mixture was then centrifuged for five minutes at approximately 12,000 x g on a table top centrifuge. Serum was pipetted off of the erythrocyte pellet into clean microcentrifuge tubes and serum samples were stored at 4ºC for use within the next 5 days, or placed at -20ºC for extended storage.

Hemagglutination was performed using 0.4% turkey erythrocytes and four hemagglutinating units of influenza virus A/Wyoming/03/2003. The A/Wyoming 03/2003 virus was obtained from the Centers for Disease Control (Atlanta, GA) and grown in eggs prior to use. A titer ≥ 1:40 was considered positive.

Hemagglutinin ELISA:

The hemagglutinin ELISA was preformed at AlphaVax, Inc. (Research Triangle Park, NC). Serum samples collected from pigs were sent on ice to AlphaVax. To measure antigen-specific humoral immune responses to HA in animals immunized with the HA-VRP vaccine, purified recombinant HA antigen derived from A/Wyoming/03/2003 (Protein Sciences Inc) was used in an ELISA. Briefly, 96-well Maxisorp ELISA plates (Nunc, Naperville, IL) were coated with 50 ng/well of recombinant HA protein in carbonate buffer. After overnight incubation at 4ºC, unbound antigen was discarded and each well was incubated for 1 hr with blocking buffer (PBS containing 3% bovine serum albumin) at room temperature. After washing 6 times with PBS, test sera that were serially 2-fold diluted in diluent buffer (PBS with 1% BSA and 0.05% Tween-20) were added in triplicate to antigen-coated wells. Antigen-coated wells that received no serum served as background controls. Plates were incubated for 1 hour at room temperature, then rinsed six times with PBS. Alkaline phosphatase-conjugated anti-pig IgG (whole molecule) antibody (Sigma, St. Louis, MO) was added to each well and incubated for 1 hr at room temperature. Wells were rinsed again six times with PBS before addition of alkaline phosphatase substrate and chromogen. Samples were read at 405 nm on a PowerWave 200 Microplate Scanning Spectrophotometer (BioTek Instruments, Winooski, VT). Endpoint titers were calculated using SOFTmax (Molecular Device Corp., Menlo Park, CA), and mean antibody titers were determined for each group. Titers are reported as the reciprocal of the serum dilution at which the A_{405} is ≥ 0.2.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

RT-PCR was performed on nasal swabs to detect influenza A virus (H3N2) antigen. Nasal swab samples collected in 1mL sterile PBS supplemented with 0.2% gentamicin (100mg/mL) and 2% penicillin-streptomycin solution (10,000units/mL) were removed from the -80ºC freezer and thawed. Samples were vortexed for 30 seconds two times, then swabs were removed from the samples and samples were centrifuged for five minutes at approximately 12,000 x g on a table top centrifuge. Most of the supernatant was removed from the sample, except for approximately 100µL of fluid along with any pellet that may have formed. Total RNA was then extracted from each 100µL sample using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the protocol "Purification of Total RNA from Animal Cells Using Spin Technology." For the first step, 400µL of RLT buffer was added to each sample, then vortexed for 20 seconds to homogenize the sample. The protocol was then followed from step two according to manufacturer's instructions. RNA samples were eluted in 30µL of RNase-free water. A positive control consisting of 50µL of influenza A/Wyoming/03/2003 and negative control consisting of buffers from the RNeasy Mini Kit were extracted with the samples.

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as follows. Previously published primers specific for the HA gene, subtype H3, of influenza A viruses[140] were used to amplify the HA gene of influenza. Primers were provided by Dr. Pravina Kitikoon, Thacker Lab, Iowa State University, Ames, Iowa. Reverse transcriptase PCR was set up in 25µL reactions using the Qiagen OneStep RT-PCR kit according to manufacturer's instructions. Five microliters of RNA was used for each reaction. RT-PCR was performed using the GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA). Cycling conditions were as follows: 1 cycle at 50ºC for 30 minutes; 1 cycle at 95ºC for 15 minutes; 40 cycles at 94ºC for 1 minute, 55ºC for 45 seconds, 72ºC for 2 minutes; 1 cycle at 72ºC for 10 minutes; infinite hold at 4ºC.

DNA samples were run in an E-Gel, 2% agarose (GP) (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. DNA was visualized using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Histopathology and Immunohistochemistry:

Histopathology and immunohistochemistry were performed by Dr. Bruce Janke (Iowa State University, Ames, IA) as previously described[92].

Statistics:

Tukey-Kramer HSD analysis was used to compare HI titers of pigs by group for each day that blood was collected. In order to allow for log transformation of titers with a value of 0, an arbitrary value of 0.5 was added to all HI titers, and then titers were $\log_{10}$ transformed, and analysis was preformed on log transformed data. Analysis was conducted using the statistical software JMP 6.0.0 (SAS Institute, Cary, NC).

Results

In our trial, 4 of 30 pigs had an initial maternal HI antibody titer of 1280 against influenza A/Wyoming/03/2003, while the other 26 pigs tested negative (titers = 0) for maternal antibody by HI assay (data not shown). Pigs with maternal antibody were placed in a single group (Group 5, pigs vaccinated with PRRS lysate + V3014 HA VRP) and the other pigs were randomly assigned to the remaining five groups. Figure 1 shows the geometric mean titers of pigs by group for Groups 1-4.

Data from groups 1, 2, 3, 4, and 6 were used in the statistical analysis for this trial. Data from Group 5 was omitted from analysis due to the fact that 4 of the 5 pigs in Group 5 were not randomly assigned to the group. There was no significant difference in HI titers between any of the five groups analyzed on any of the days using Tukey-Kramer HSD

analysis. However, results of the statistical analysis were suggestive that the group administered HA protein + Emulsigen-D adjuvant (Group 4) had significantly higher HI titers than any of the other groups (data not shown), but further work is needed to confirm this suggestive data.

Figures 2, 3, 4, and 5 show HA ELISA titers on Days 56 and 68 for pigs vaccinated intramuscularly (IM) with influenza HA protein lysate alone, HA protein lysate plus MVP Emulsigen-D adjuvant, PRRS virus protein lysate plus HA-VRP, or HA protein lysate plus PRRS-VRP, respectively. ELISA OD_{405} values from serum of pigs collected on Day -3 were used as baseline or "negative" values to calculate ELISA titers on Days 56 and 68. Pigs 133, 134, and 135 had maternal antibody against A/Wyoming with ELISA titers of > 40 on Day -3 (Figure 9). The other 27 pigs had HA ELISA titers of < 40 on Day -3. Pig 136 was shown to have maternal antibody by HI assay, but not by ELISA. HA ELISA titers were negative (titers < 40) for all pigs vaccinated with PRRS protein lysate alone, or with PRRS protein lysate plus MVP Emulsigen-D adjuvant (groups 1 and 3) on both Days 56 and 68 (data not shown).

In Figure 2, one pig of five vaccinated with HA protein lysate alone demonstrated a response to vaccination by ELISA by Day 56, and two pigs of five demonstrated a response by Day 68, but responses in both pigs were low (titers ≤ 80) when compared to other groups of pigs in the trial. In Figures 3, 4, and 5, five of five pigs in all three groups demonstrated an ELISA response to vaccination on both days (titers ≥ 80). Pigs vaccinated with HA protein lysate plus MVP Emulsigen-D adjuvant (Figure 3) had the highest ELISA titers of all groups in Trial I, with titers of all pigs ≥ 1280 on both Days 56 and 68.

Figure 6 shows the western blot of the HA protein used to vaccinate pigs in Trial I. Total protein in each sample was determined (Table 2) using the RC DC Protein Assay kit

according to manufacturer's instructions (BioRad, Hercules, CA). Protein bands at 75 kDa show the location of the HA0 molecule (Figure 6).

Table 3 shows western blot data from serum or bronchoalveolar lavage (BAL) fluid from pigs in each group. Samples were tested for the presence of antibodies against A/Wyoming/03/2003. Pigs vaccinated with PRRS lysate alone, with V3014 HA lysate alone, or with PRRS lysate plus MVP Emulsigen-D adjuvant had no detectable antibody against A/Wyoming HA protein in both serum and BAL fluid on Days 56 and 68. Two of five pigs had detectable serum antibody on Day 68 in pigs vaccinated with HA lysate plus PRRS-VRP, but no antibody was detected in the serum of these pigs on Day 56 or in the BAL fluid on Day 68. Antibody was detected in the serum of all five pigs vaccinated with HA lysate plus MVP Emulsigen-D adjuvant and all five pigs vaccinated with PRRS lysate plus HA-VRP on both Days 56 and 68. Antibody was detected in the BAL fluid of all five pigs vaccinated with HA lysate plus MVP Emulsigen-D adjuvant and four of five pigs vaccinated with PRRS lysate plus HA-VRP on Day 68.

Histopathology and immunohistochemistry on lung tissues samples from all pigs in this trial were performed by Dr. Bruce Janke (Iowa State University, Ames, Iowa). No conclusive lesions were observed in any of the lung tissue samples from any pigs in any of the groups. Similarly, no virus was detected in lung tissue samples via immunohistochemistry in any pigs in this trial.

No live virus was detected by virus isolation in MDCK cells in any of the bronchoalveolar lavage fluid samples collected at necropsy from any pigs in this trial. Table 4 shows RT-PCR data from nasal swabs collected from pigs 1, 2, and 3 days post challenge. At 1 day post challenge (Day 64), virus was detected in the nasal swabs from 4 of 30 pigs, 2 pigs who had been vaccinated with HA protein lysate alone (pigs 117 and 129), and 2 pigs

who had been vaccinated with PRRS protein lysate plus V3014 VRP (pigs 133 and 135). At 2 days post challenge (Day 65), virus was detected in the nasal swabs from 2 of 30 pigs, 1 pig who had been vaccinated with HA protein lysate alone (pig 117), and 1 pig who had been vaccinated with HA protein lysate plus PRRS VRP (pig 125). At 3 day post challenge (Day 66), virus was detected in the nasal swabs from 6 of 30 pigs, 2 pigs who had been vaccinated with HA protein lysate alone (pigs 117 and 129), 2 pigs who had been vaccinated with PRRS protein lysate plus MVP Emulsigen-D adjuvant (pigs 122 and 126), and 2 pigs who had been vaccinated with PRRS protein lysate plus V3014 VRP (pigs 133 and 135).

Figure 7 shows average body temperatures of pigs, by group, from one day before and four days after virus challenge. Average temperatures for groups 3 and 4 declined from the first day temperatures were taken (one day prior to challenge) to two days after challenge. Groups 1, 2, 5, and 6 all showed a slight increase in temperature from one day prior to challenge to one day after. Pigs in all groups maintained a steady average temperature from two to four days post challenge.

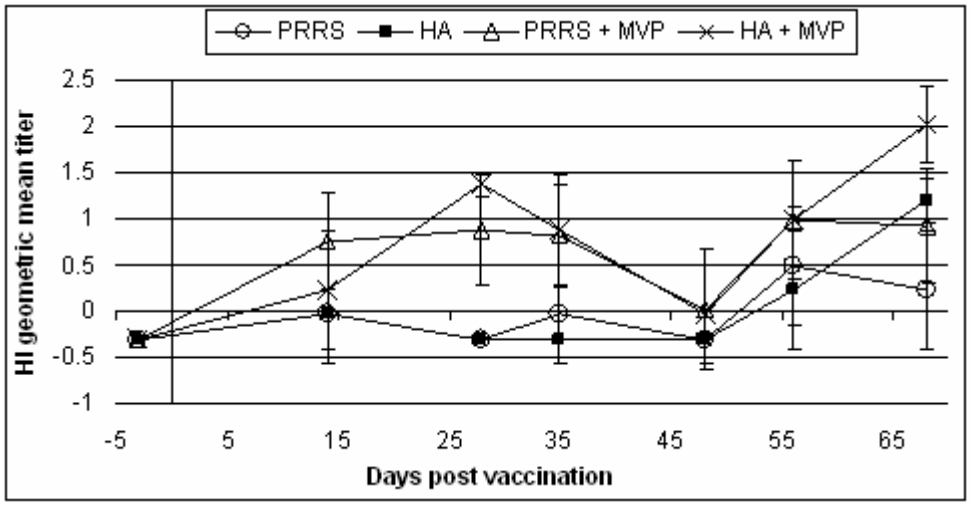

Figure 1: Trial I - Hemagglutination-Inhibition geometric mean titers (GMT) of pigs by group. Group 1: PRRS, Group 2: HA, Group 3: PRRS + MVP, Group 4: HA + MVP, Group 5: not shown, Group 6: not shown. GMT were calculated by taking the $\log_{10}$(HI titers + 0.5) of pigs on all days, then calculating the mean of the logs. Error bars represent 95% confidence interval.

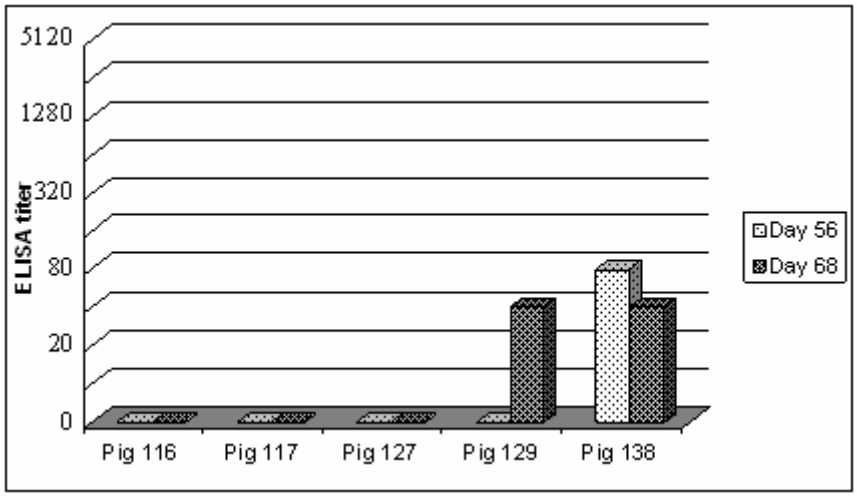

Figure 2: HA ELISA titers for Trial I, group 2, pigs vaccinated intramuscularly in the ham with ≤ 25 µg V3014 HA protein lysate on Days 0, 21 and 49. ELISA titers are defined as the reciprocal of the highest dilution of serum for which the optical density was ≥ 2 times the optical density of serum diluted 1:40 from Day -3.

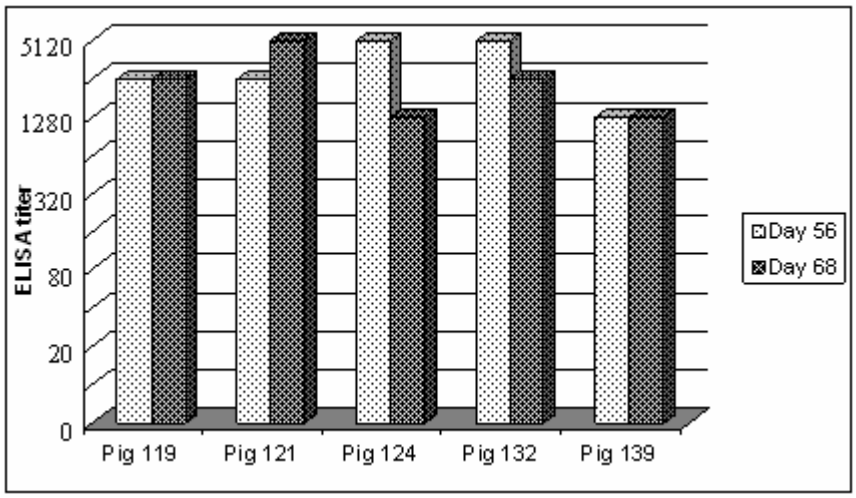

Figure 3: HA ELISA titers for Trial I, group 4, pigs vaccinated intramuscularly in the ham with ≤ 25 µg V3014 HA protein lysate plus MVP Emulsigen-D adjuvant on Days 0, 21 and 49. ELISA titers are defined as the reciprocal of the highest dilution of serum for which the optical density was ≥ 2 times the optical density of serum diluted 1:40 from Day -3.

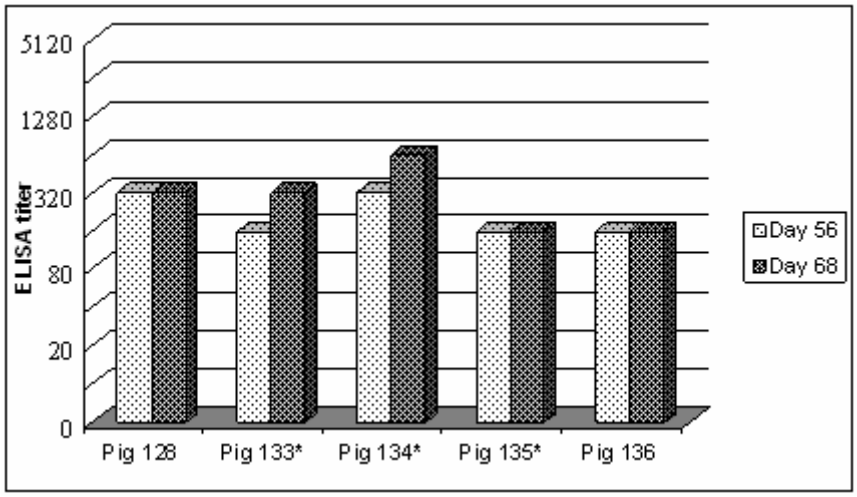

Figure 4: HA ELISA titers for Trial I, group 5, pigs vaccinated intramuscularly in the ham with ≤ 25 µg PRRS virus protein lysate (negative control) plus 2.1 x 10^8 IU/mL of V3014 HA-VRP on Days 0, 21 and 49. Pigs 133, 134, and 135 had an ELISA titer of >40 on Day -3 (considered cutoff point for positive titer), but exact titers were not determined for these pigs on Day -3. ELISA titers are defined as the reciprocal of the highest dilution of serum for which the optical density was ≥ 2 times the optical density of serum diluted 1:40 from Day -3.

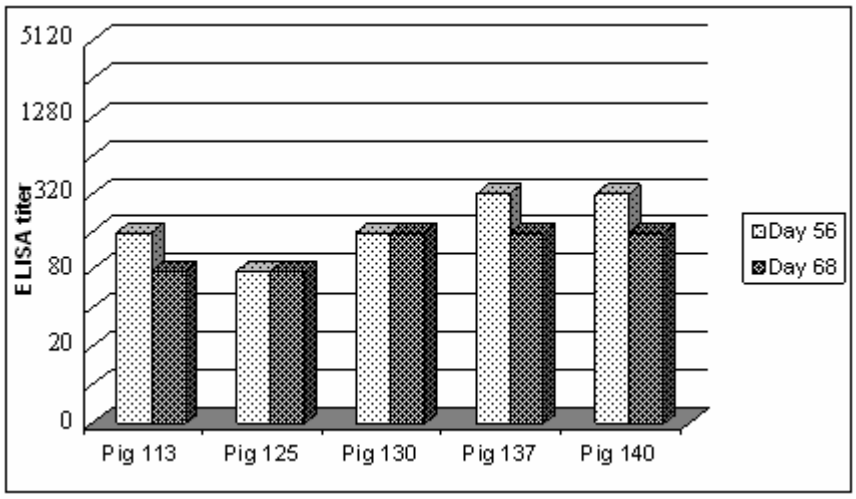

Figure 5: HA ELISA titers for Trial I, group 6, pigs vaccinated intramuscularly in the ham with ≤ 25 µg V3014 HA protein lysate plus 8.8 x 10^8 IU/mL of PRRS-VRP on Days 0, 21 and 49. ELISA titers are defined as the reciprocal of the highest dilution of serum for which the optical density was ≥ 2 times the optical density of serum diluted 1:40 from Day -3.

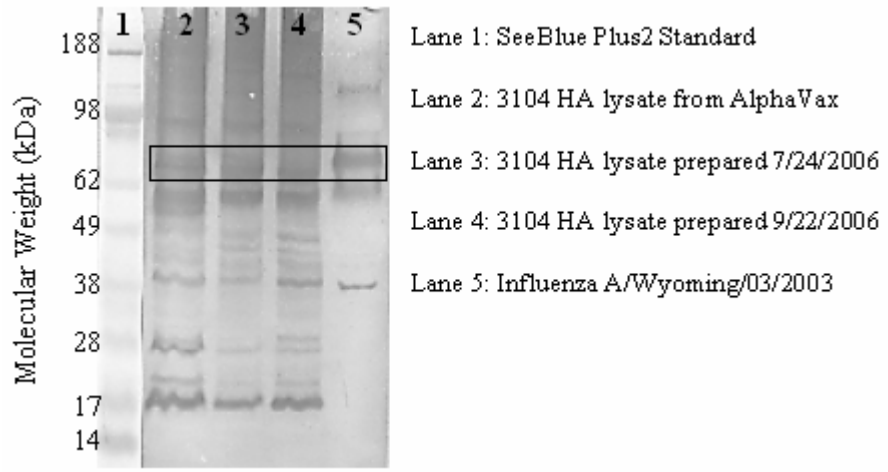

Figure 6: Western blot of influenza A/Wyoming/03/2003 HA protein lysate used as vaccine for pigs in Trial I. The influenza HA protein (HA0) is approximately 75 kDa in size (bands inside black box). V3014 HA lysate from AlphaVax was used to vaccinate pigs on Day 0. V3014 HA lysate prepared 7/24/2006 was used to vaccinate pigs on Day 21. V3014 HA lysate prepared 9/22/2006 was used to vaccinate pigs on Day 49.

Table 2: Total protein concentration of HA lysate used to vaccinate pigs in Trial I.

Sample	Total Protein (mg/mL)
V3014 HA lysate from AlphaVax	1.112
V3014 HA lysate prepared 7/24/2006	0.577
V3014 HA lysate prepared 9/22/2006	0.581

Table 3: Trial I western blot data showing presence or absence of antibodies against A/Wyoming hemagglutinin in serum or BAL fluid of pigs.

Group # (5 pigs/ group)	Subunit Treatment	Day 56[a] HA antibody in serum	Day 68[b] HA antibody in serum	Day 68[c] HA antibody in BAL fluid
1	PRRS lysate	0/5	0/5	0/5
2	V3014 HA lysate	0/5	0/5	0/5
3	PRRS lysate + MVP adjuvant	0/5	0/5	0/5
4	V3014 HA lysate + MVP adjuvant	5/5	5/5	5/5
5	PRRS lysate + V3014 HA VRP	5/5	5/5	4/5
6	V3014 HA lysate + PRRS VRP	0/5	2/5	0/5

[a,b,c] Serum or bronchoalveolar lavage (BAL) fluid samples from pigs vaccinated with either PRRS lysate or HA lysate, with or without adjuvant, were tested via western blot for the presence of HA-specific antibodies. Values are reported as number of pigs positive for HA antibody/ total number of pigs per group.

Table 4: Trial I RT-PCR data showing presence or absence of A/Wyoming antigen in nasal swabs of pigs 1-3 days post challenge.

Group # (5 pigs/ group)	Subunit Treatment	Day 64[a] Pigs Positive for Viral RNA	Day 65[b] Pigs Positive for Viral RNA	Day 66[c] Pigs Positive for Viral RNA
1	PRRS lysate	Negative	Negative	Negative
2	V3014 HA lysate	117 129	117	117 129
3	PRRS lysate + MVP adjuvant	Negative	Negative	122 126
4	V3014 HA lysate + MVP adjuvant	Negative	Negative	Negative
5	PRRS lysate + V3014 HA VRP	133 135	Negative	133 135
6	V3014 HA lysate + PRRS VRP	Negative	125	Negative

[a,b,c] RT-PCR was performed on RNA extracted from nasal swabs of pigs 1, 2, and 3 days post-challenge (Days 64, 65, and 66). "Negative" indicates that no virus was detected in any of the pigs on that day via RT-PCR. Pig number indicates that a pig did contain virus in its nasal swab via RT-PCR.

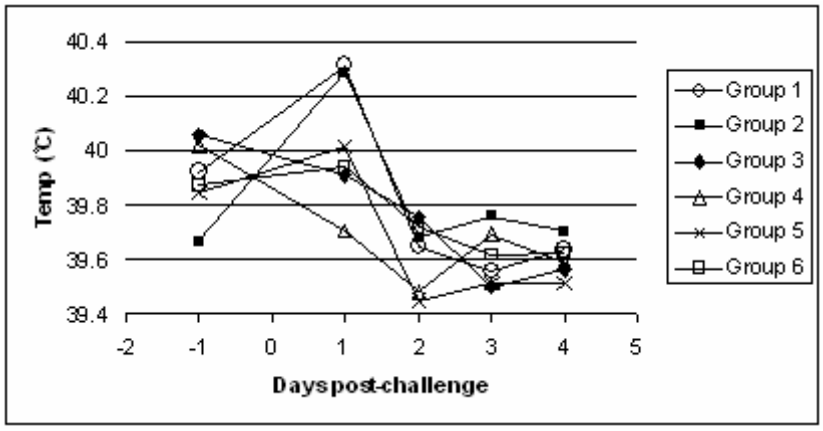

Figure 7: Trial I - Group average body temperatures 1 day before and 4 days post-challenge.

Discussion

Our purpose for Trial I was to evaluate the ability of recombinant hemagglutinin (rHA) protein produced *in vitro* in Vero cells from alphavirus replicons to elicit an immune response in pigs. We also wanted to evaluate the ability of a response elicited by replicon-

derived HA protein to protect pigs from challenge with live A/Wyoming/03/2003 influenza virus. Based upon hemagglutination-inhibition (HI), ELISA, and western blot data, we have suggestive evidence that protein derived from Venezuelan equine encephalitis virus replicon particles is able to elicit an immune response in pigs. Based upon HI data, development of an immune response in pigs to the rHA protein took longer than expected. This delay in response may be due to a number of factors including amount of rHA protein administered to each pig and dosing schedule. The amount of rHA protein in the first dose was 25µg of protein as determined at AlphaVax by densitometry analysis. However, the exact amount of rHA protein for the second and third vaccinations was not determined. Figure 6 shows that rHA protein was present in all vaccination formulations, but Table 2 shows that total protein was lower for the second and third vaccinations; therefore the amount of rHA protein administered to those pigs was most likely less than 25µg. In humans, a dose of 45µg of rHA protein produced from baculovirus vectors has been selected to use as a commercial vaccine[66]. It is possible that a larger amount of rHA was needed to elicit a stronger and more rapid immune response in pigs.

Influenza A/Wyoming/03/2003 was used as the human vaccine strain for the 2004-2005 flu season[44]. Replicons expressing the HA gene of this virus had previously been constructed by AlphaVax Inc., therefore these replicons were used for initial testing in pigs. It was not known if A/Wyoming would be able to efficiently infect and cause disease in pigs. Based upon histopathology, immunohistochemistry, and viral titrations from bronchoalveolar lavage (BAL) fluid, it does not appear that the virus was able to cause disease in these pigs. Reverse transcriptase PCR results from Table 4 demonstrate that A/Wyoming was able to replicate, even if at a low level, in pigs. The negative control pigs vaccinated with PRRS protein lysate alone showed no viral shedding in the nasal swabs. Because of these results, we were not able to determine if the immune response elicited by the rHA protein was able to protect pigs from virus challenge. It cannot be determined from this trial if the A/Wyoming

virus is able to cause disease in pigs for several reasons. At the time of virus challenge, pigs were 12 weeks old and weighed approximately 100 to 120 pounds. It is difficult to cause clinical disease in pigs of this size, and pigs weighing around 50 to 75 pounds are preferred for demonstrating lung lesions and viral shedding after challenge[117]. Also, ten milliliters of virus at a concentration of 10^6 $TCID^{50}$/mL was injected into each pig's lungs, and due to the large size of the pigs at time of challenge, more virus may have been needed to cause clinical disease.

CHAPER 5: GENERAL DISCUSSION

The work presented in this thesis describes the first time viral replicon particles derived from Venezuelan equine encephalitis virus (VRPs) have been evaluated for use in pigs. As VRPs had never been used in pigs to express a foreign gene, we wanted to determine if the replicons could express a gene of interest that would elicit an immune response in pigs. We also wanted to know if protein produced *in vitro* from VRPs would be able to elicit an immune response in pigs, either alone or in combination with an adjuvant.

From the data described in Chapter 3 of this thesis, it has been shown that VRPs can elicit an immune response to a gene of interest in pigs. Two separate trials were conducted in which VRPs expressing the influenza hemagglutinin (HA) gene were evaluated in pigs. In Trial I, we used VRPs derived from Venezuelan equine encephalitis virus (VEE) strain V3014 to vaccinate pigs intramuscularly with different concentrations of the replicon. Due to the presence of maternally derived antibodies (MDA) against A/Wyoming in 23 of 24 pigs, further work was needed to determine if the response seen in this one pig was representative of a larger portion of the swine population. Other pigs in the study did appear to respond to vaccination following decay of their MDA (data not shown) and more pigs may have responded if dosing schedule and amount of VRP administered were optimized.

In Trial II, we used VRPs derived from either V3014 or TC-83 to vaccinate pigs intramuscularly or intranasally against A/Wyoming/03/2003. Based upon hemagglutination-inhibition (HI) and ELISA titers, we showed that the intramuscular (IM) route of vaccination was more effective at eliciting an immune response against HA than the intranasal (IN) route when vaccinating with VRPs. We also showed that VRPs derived from either TC-83 or V3014 appeared to be equally efficient at expressing the HA protein *in vivo*, as no significant difference was observed in immune responses between pigs vaccinated with either of these

VRPs. Because V3014 is considered a select agent, VRPs derived from this VEE virus strain must be constructed under Biosafety-level 3 conditions. Replicons constructed from TC-83 may be constructed under Biosafety-level 2 conditions; therefore the use of TC-83 to construct VRPs is desirable in that production costs are less for VRPs derived from TC-83 than for VRPs derived from V3014. In addition, results from Trial II provide information to conclude that the results seen in Trial I for pig 24 (no maternal antibody against A/Wyoming) are not unique to that one animal, and can be repeated.

Although the sample size of pigs is not large enough to make any specific conclusions about whether or not VRPs can illicit an immune response in pigs against HA in the presence of MDA, it is interesting to note that most pigs in Trial II with an initial MDA titer against A/Wyoming and vaccinated either IM or IN with HA-VRPs maintained HI titers ≥ 40 throughout most or all of the study (data not shown). Pigs in the negative control Gag-VRP groups that had an initial maternal antibody titer against A/Wyoming all reached HI titers $\leq$ 20 by Day 52. Further work is needed to evaluate the ability of VRPs derived from VEE to protect pigs from influenza challenge in the presence of MDA.

Work described in Chapter 4 suggests that protein derived from VRPs expressed *in vitro* is able to elicit an immune response in pigs when administered in combination with an adjuvant. Based upon HI data, development of an immune response in pigs to the rHA protein took longer than expected. This delay in response may be due to a number of factors including amount of rHA protein administered to each pig and dosing schedule. The amount of rHA protein in the first dose was 25µg of protein as determined at AlphaVax by densitometry analysis. However, the exact amount of rHA protein for the second and third vaccinations was not determined. In humans, a dose of 45µg of rHA protein produced from baculovirus vectors has been selected to use as a commercial vaccine[66]. It is possible that a

larger amount of rHA was needed to elicit a stronger and more rapid immune response in pigs.

The Influenza A virus hemagglutinin (HA) gene was selected as the gene of interest to use in pigs for several reasons. Influenza is common respiratory pathogen that has had significant detrimental effects on the swine industry for decades, and current vaccination methods are insufficient to provide broad heterologous protection against the newly emerging reassortant virus strains. Secondly, the HA protein is found on the surface of the virus and antibody against this protein has been shown to be protective against virus challenge[66, 93, 113]. Future work may evaluate the ability of VRPs to elicit an immune response against other influenza proteins in pigs and against proteins from other swine pathogens. Many questions have arisen from work presented in this thesis. Is the immune response elicited by the VRPs or HA protein able to protect pigs from challenge with virulent influenza virus? Do VRPs have the ability to elicit a protective immune response in the presence of maternal antibody? Can VRPs be used multiple times in a pig without being inhibited by VRP neutralizing antibodies? Further research must be done in order for these questions to be answered.

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